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**Saboulard et al.**

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(54) **VIII FACTORS FOR THE TREATMENT OF TYPE A HEMOPHILIA**

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USPC ..... **514/14.1**; 435/13

(58) **Field of Classification Search**  
None  
See application file for complete search history.

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Human coagulation factor VIII precursor isoform 1 (last viewed on May 25, 2012).\*

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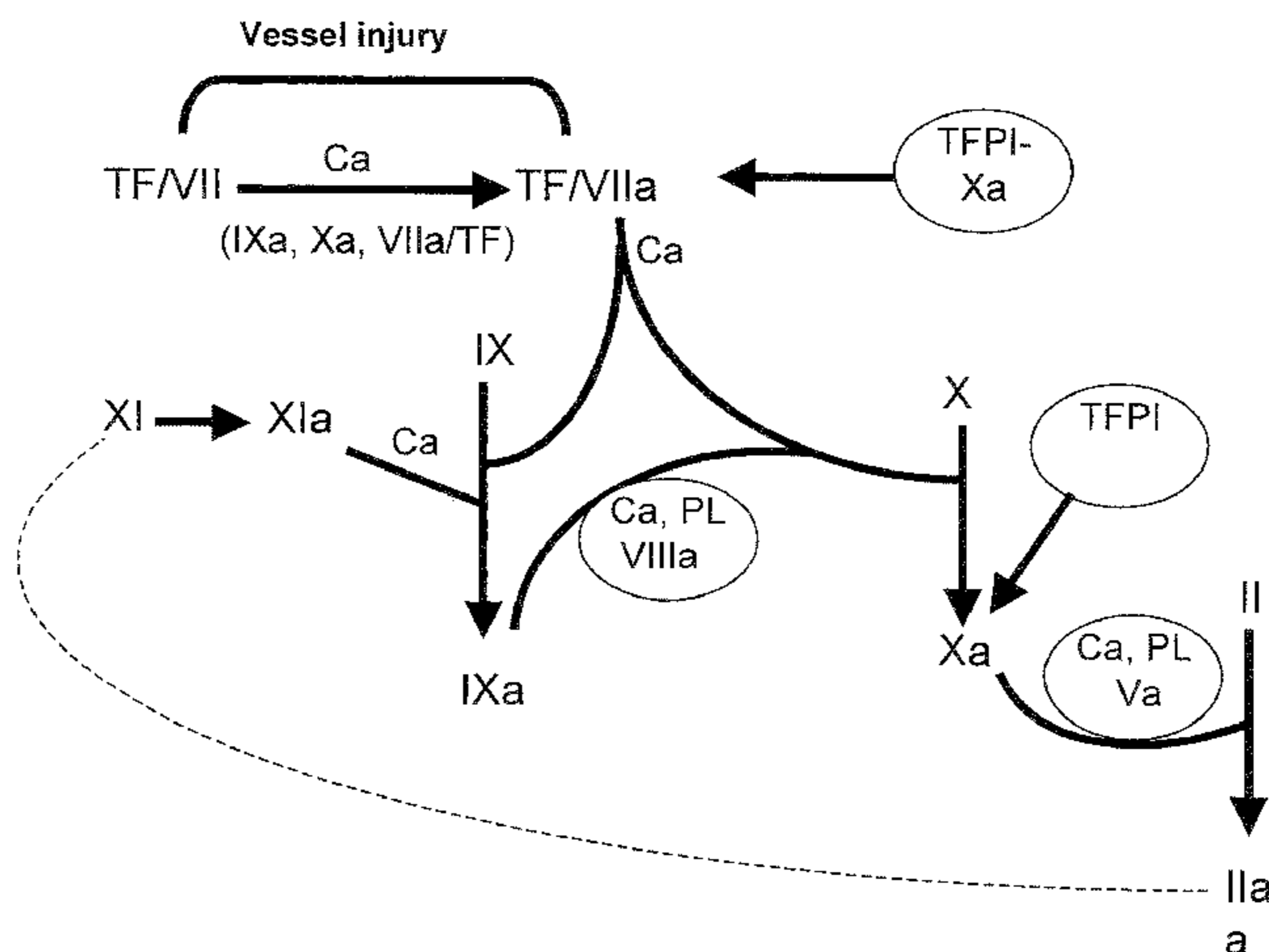
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(57) **ABSTRACT**

The present invention relates to improved human FVIII variants having at least one substitution in the A2 and/or C2 domain. The present invention also relates to their uses in the treatment of hemophilia A, particularly in patients with inhibitors.

**16 Claims, 27 Drawing Sheets**



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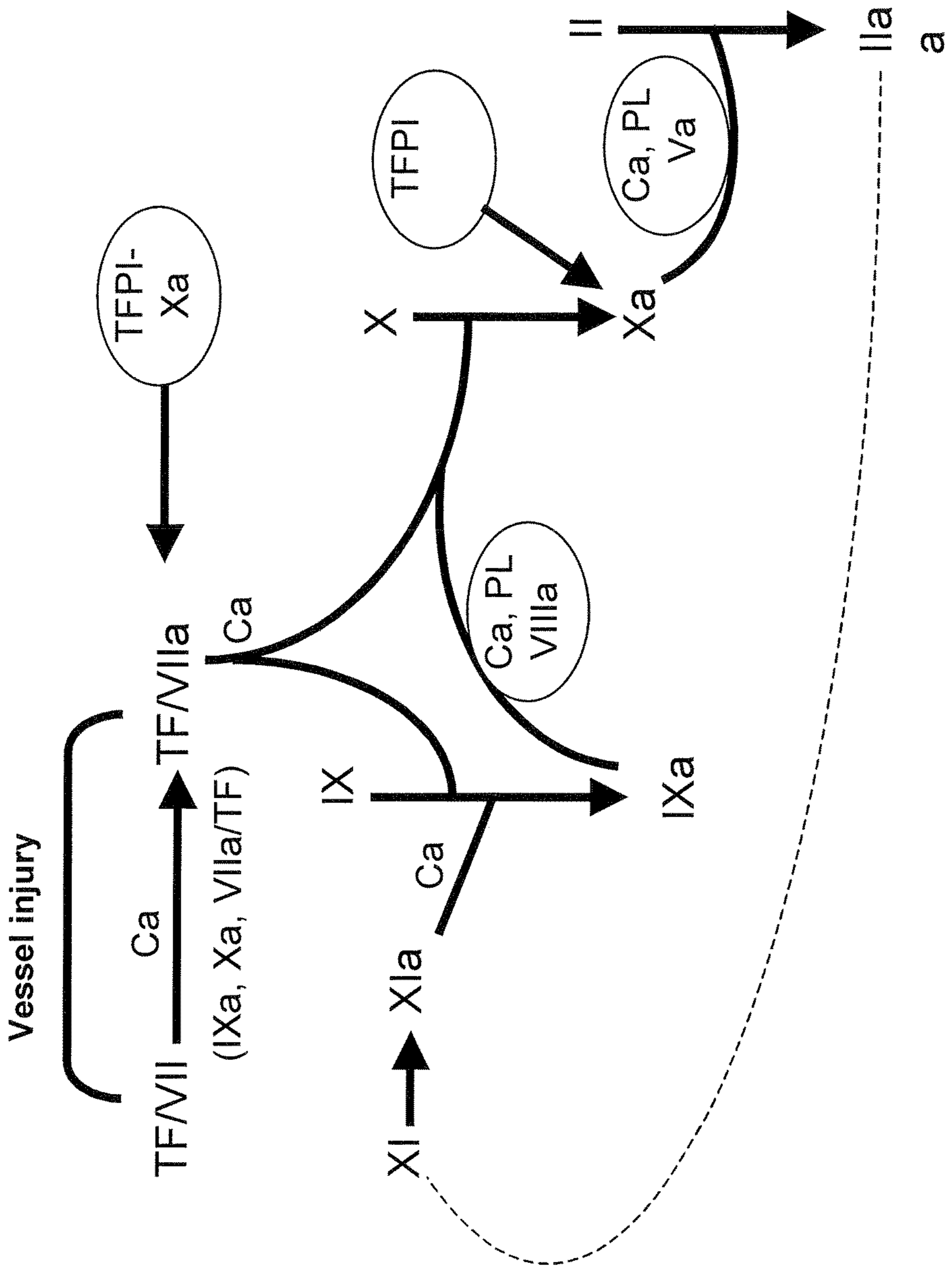


FIGURE 1

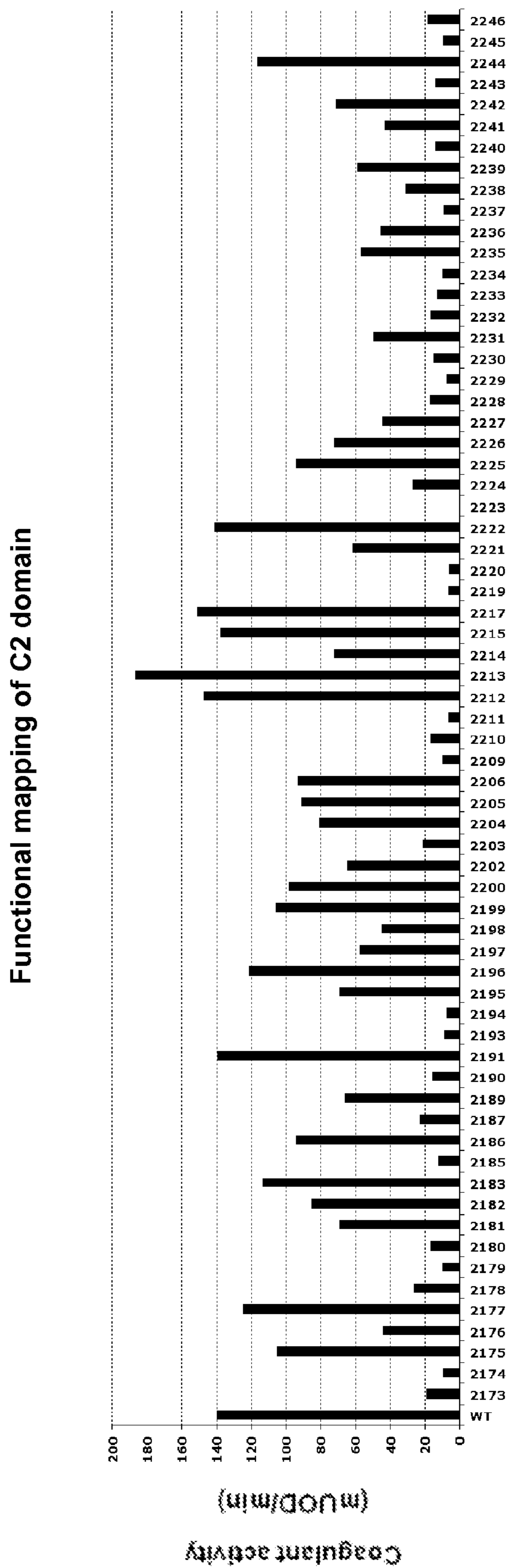


FIGURE 2A

Functional mapping of C2 domain

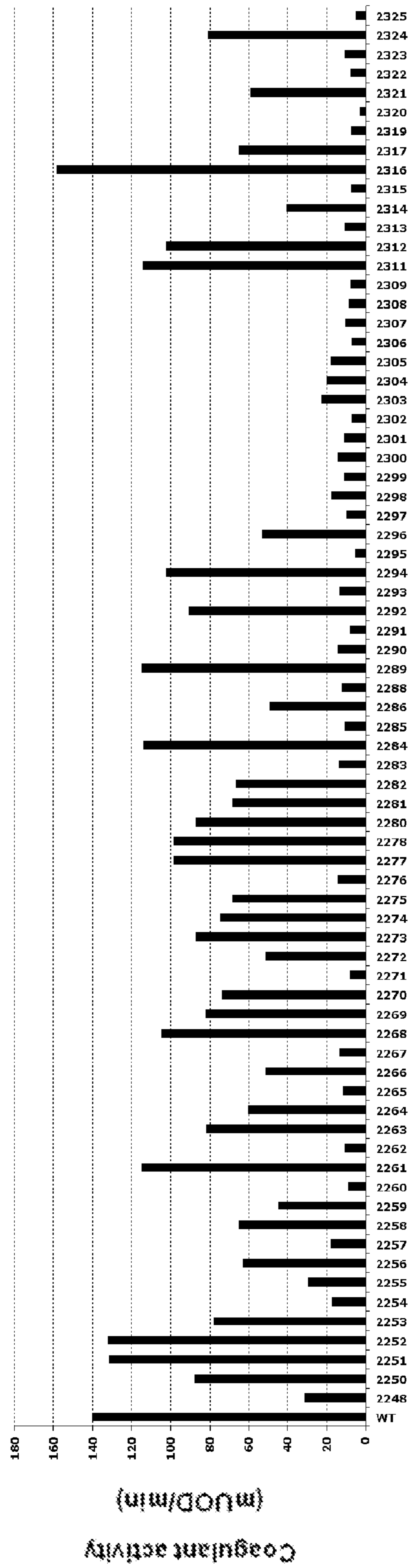


FIGURE 2B

Functional mapping of A2 domain

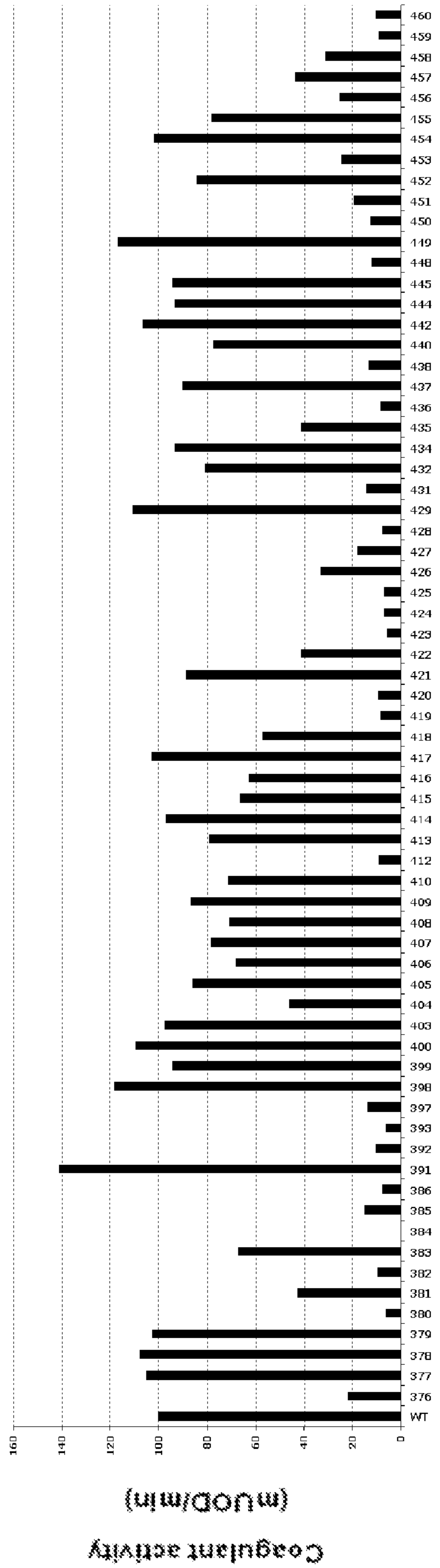


FIGURE 2C

Functional mapping of A2 domain

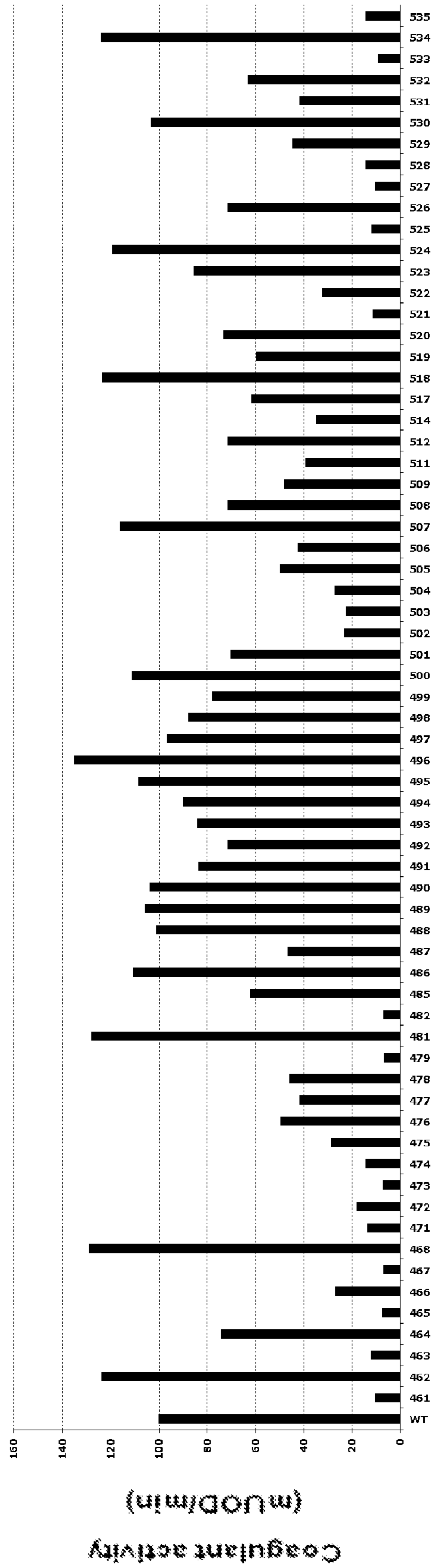


FIGURE 2D

Functional mapping of A2 domain

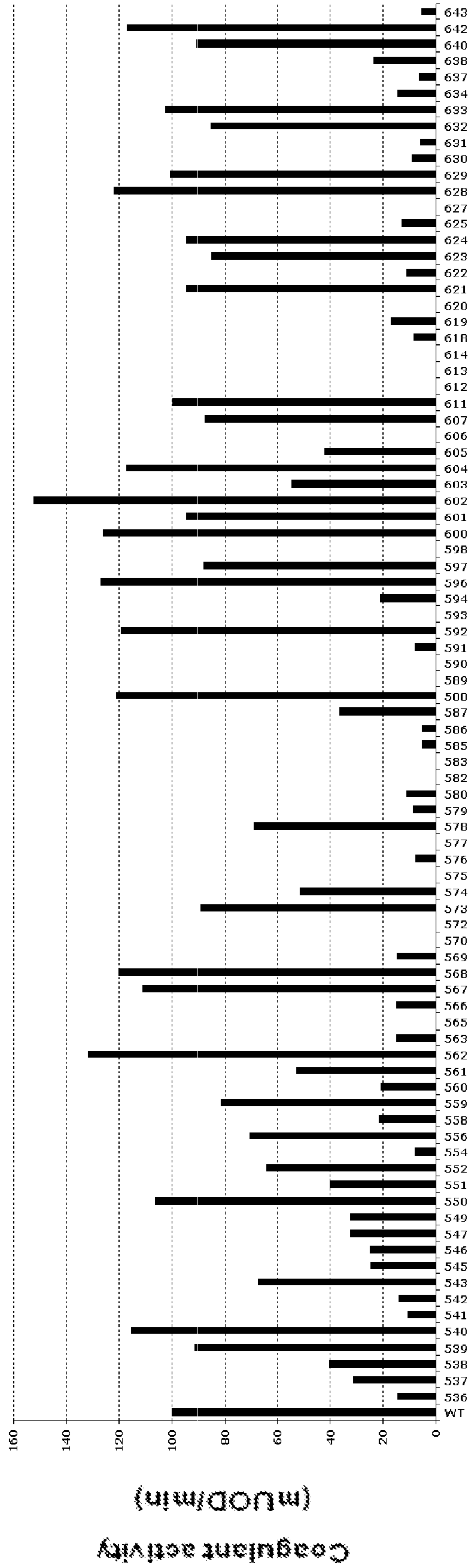


FIGURE 2E



FVIII production in culture medium

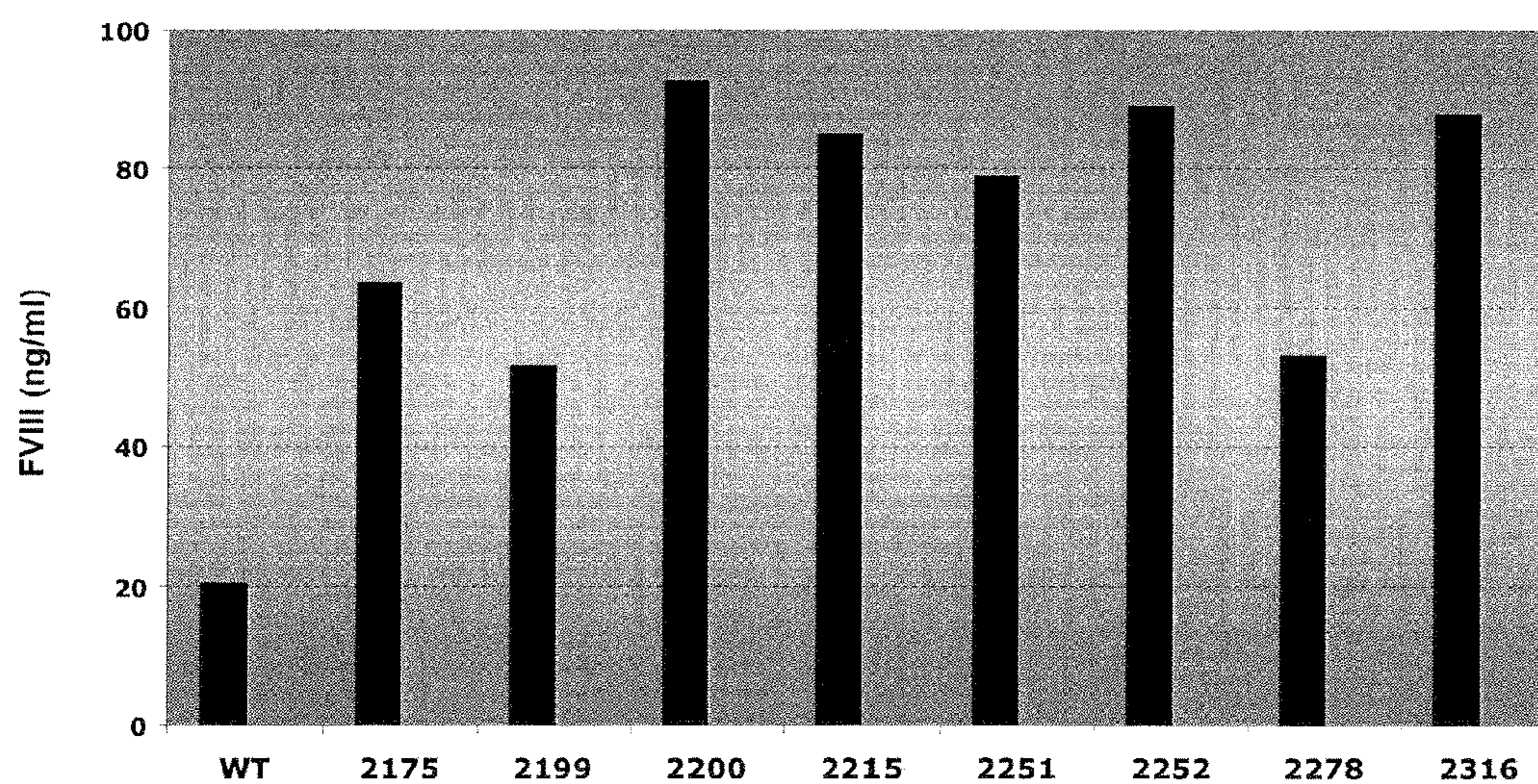
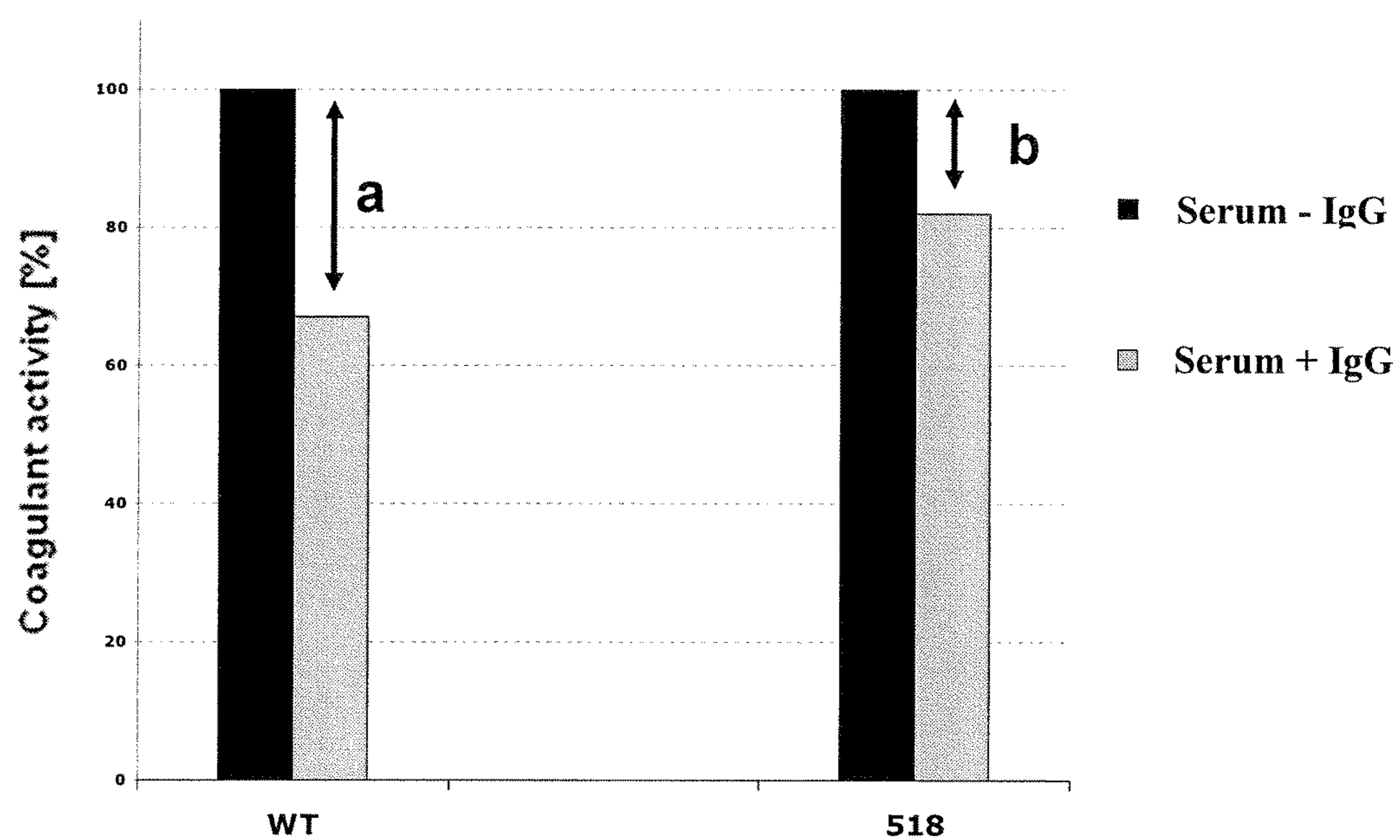


FIGURE 3

C2 mutants	Concentration higher than 10 ng/ml	Specific activity higher than 4 (mUOD/min/ng/ml)
2177	18,40	6,77
2183	11,80	9,57
2186	17,62	5,33
2191	27,27	5,11
2196	28,59	4,24
2204	14,36	5,62
2205	11,39	7,99
2206	14,62	6,36
2213	25,49	7,34
2217	24,75	6,11
2235	13,35	4,27
2258	11,61	5,60
2264	11,23	5,37
2268	11,29	9,24
2269	16,38	5,01

FIGURE 4

Example of abolition to inhibition of mutant  
518 on serum of patient TD

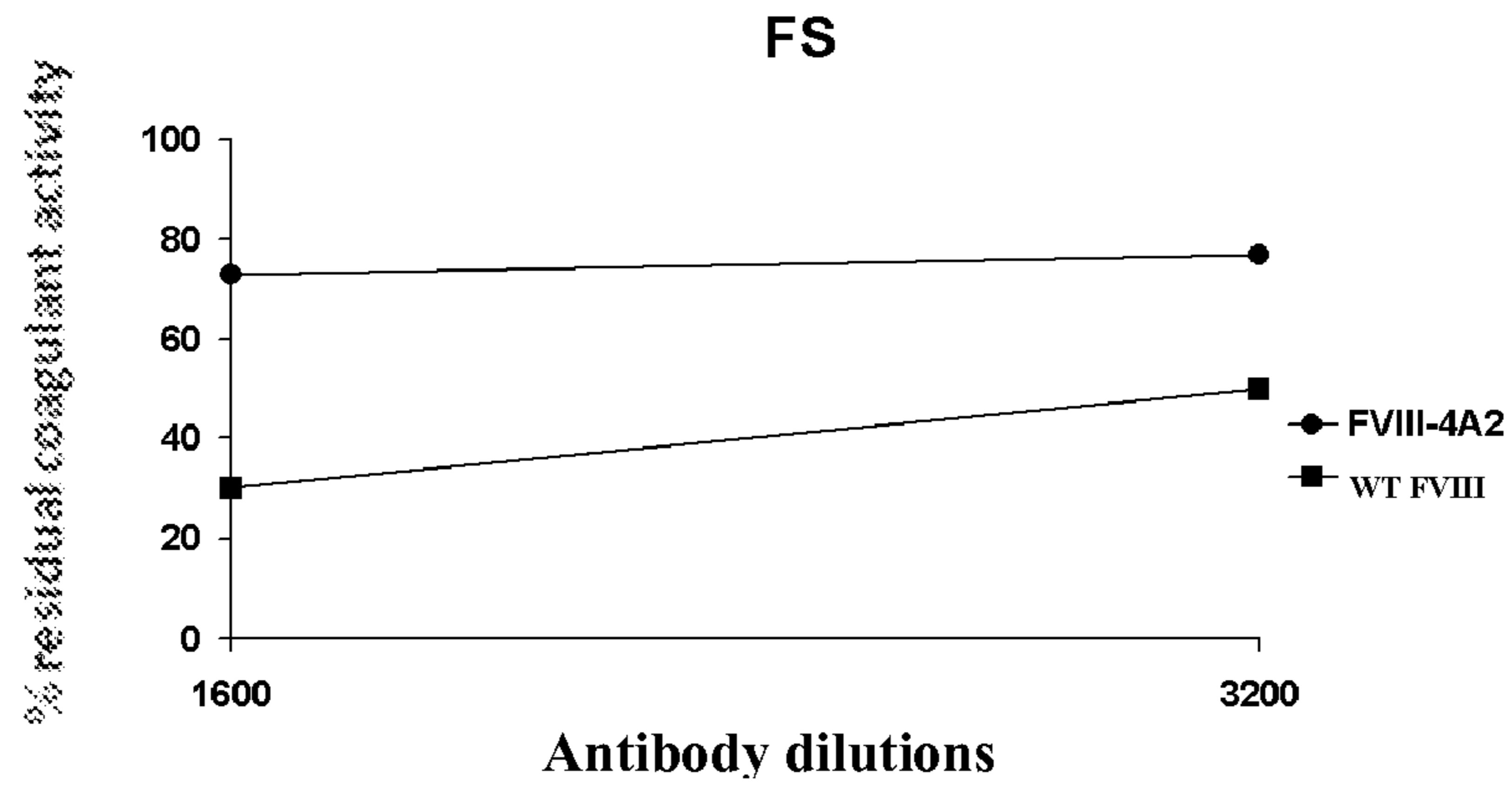


**a = % residual activity (WT)**

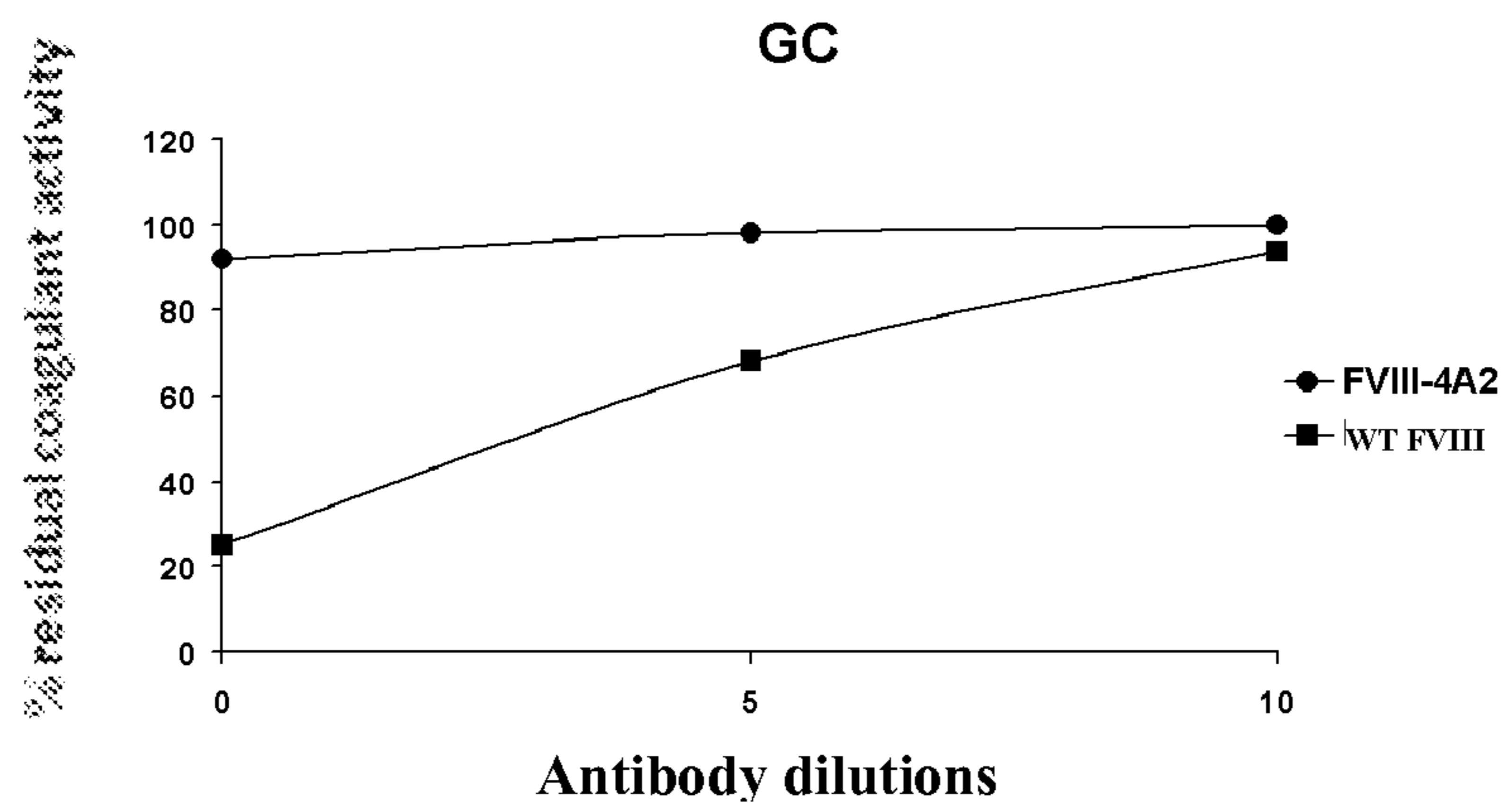
**b = % residual activity (mutant)**

**% abolition to inhibition =  $-[(b-a) / a] \times 100$**

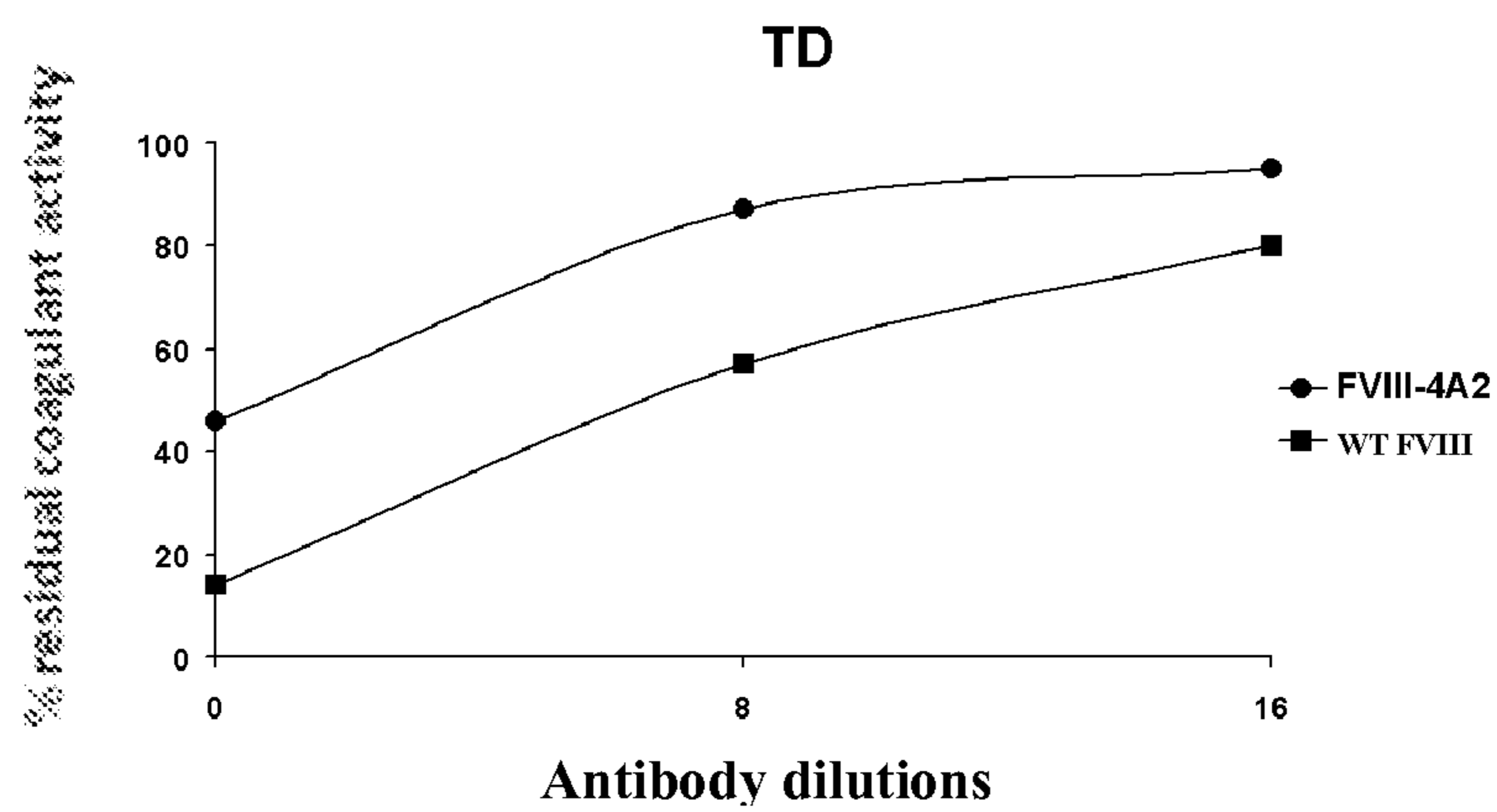
**FIGURE 5**



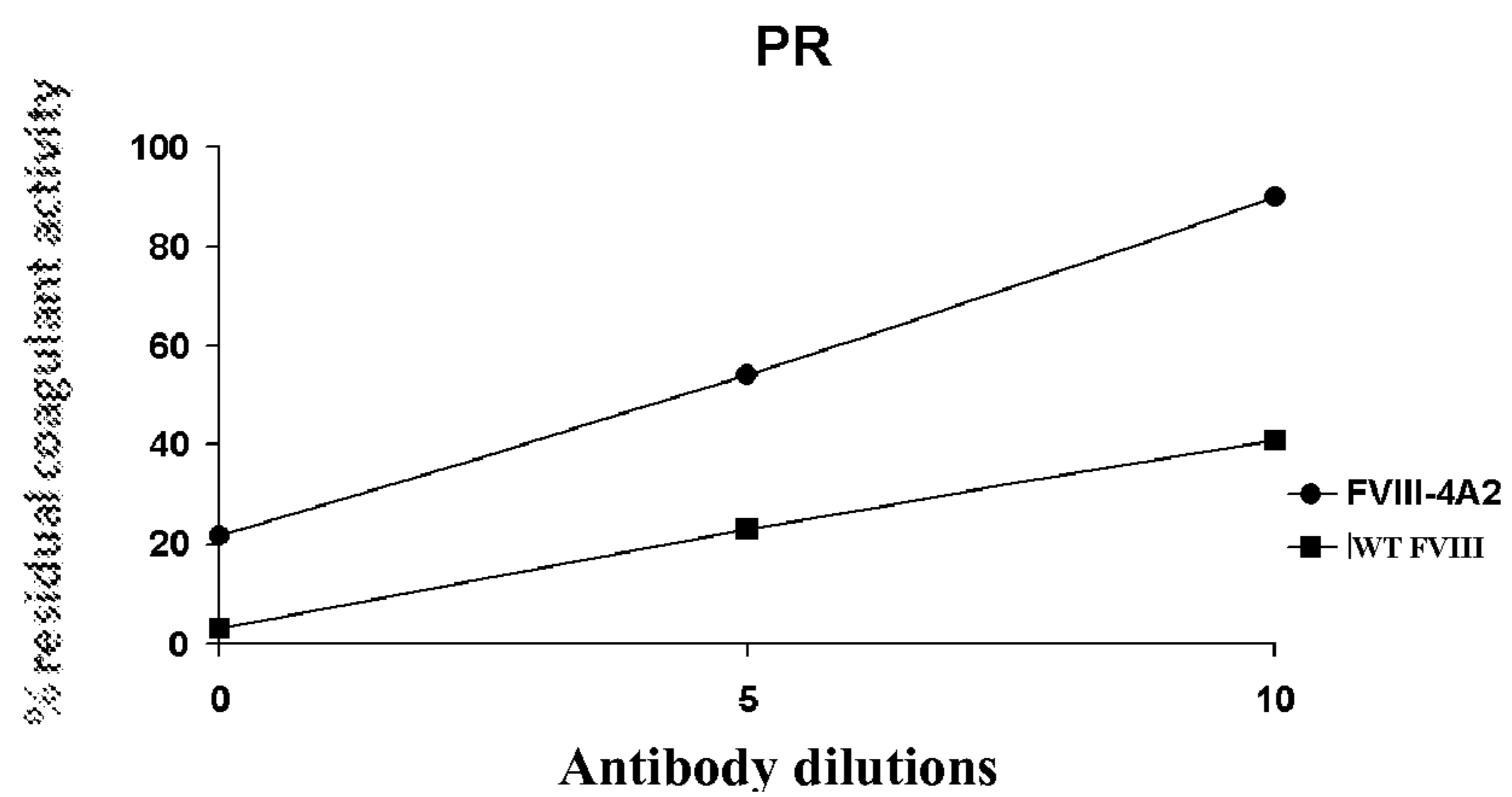
**FIGURE 6A**



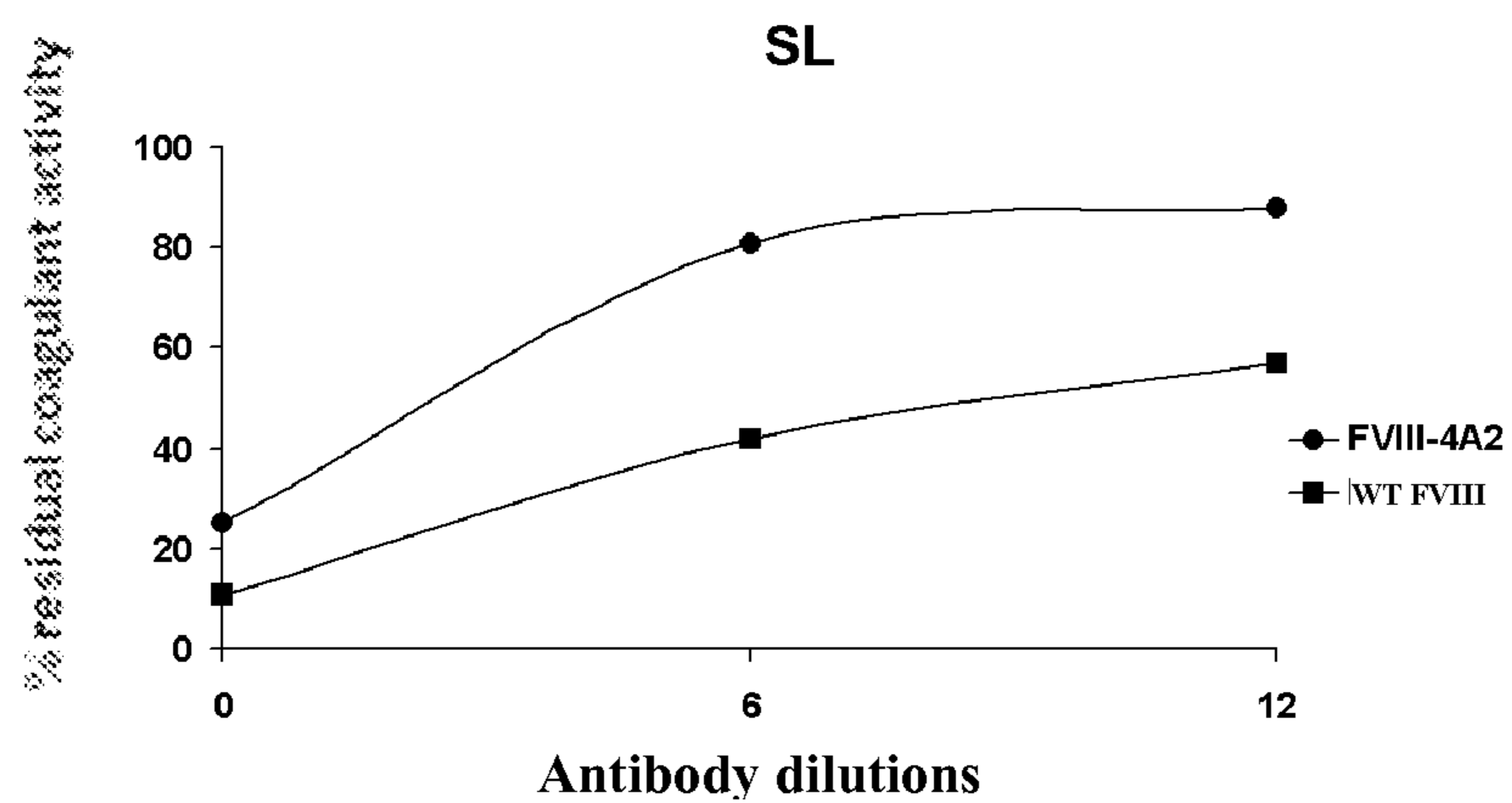
**FIGURE 6B**



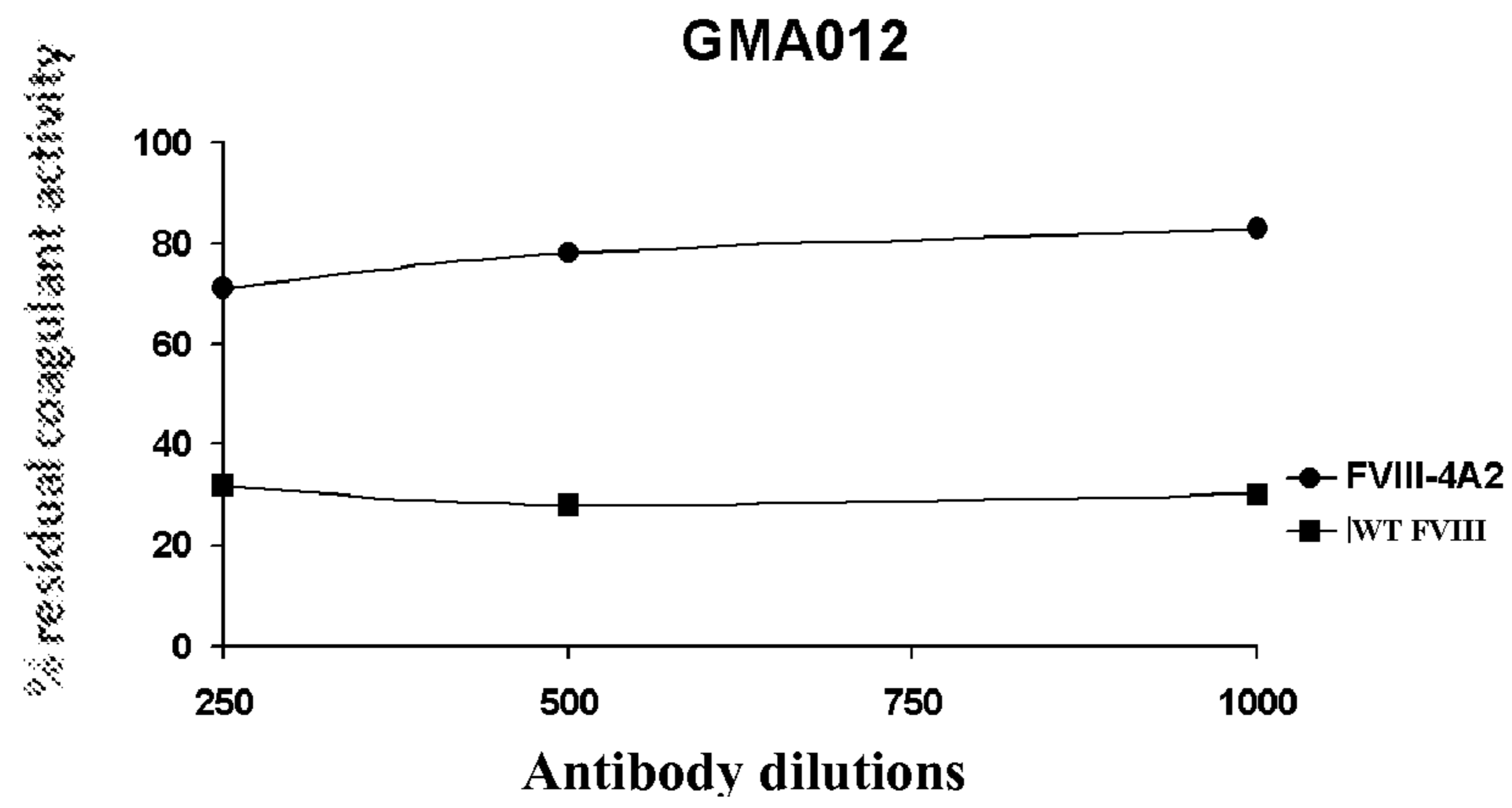
**FIGURE 6C**



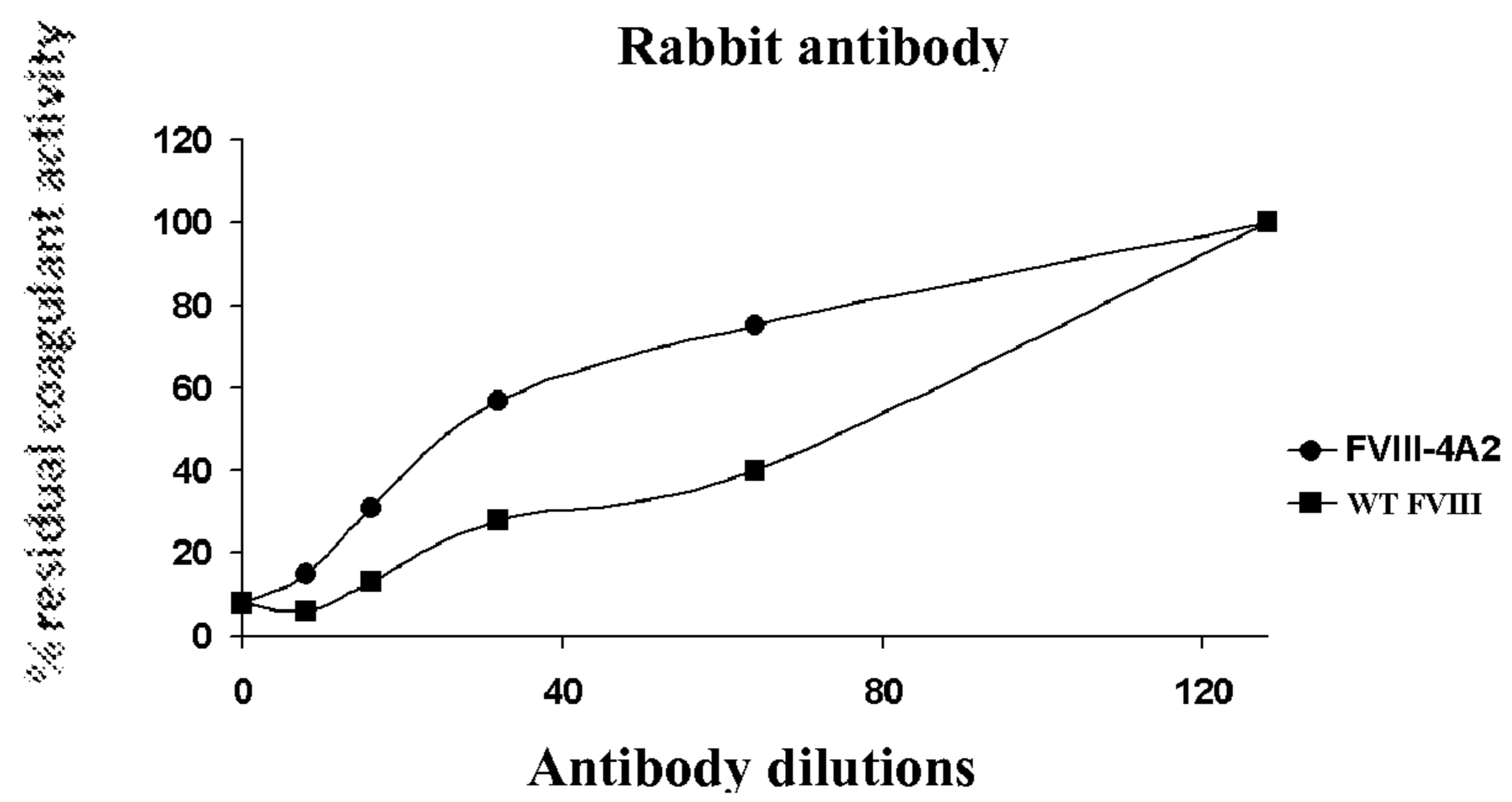
**FIGURE 6D**



**FIGURE 6E**



**FIGURE 7A**



**FIGURE 7B**

### Titration of wild-type FVIII and FVIII-4A2 by GMA-012

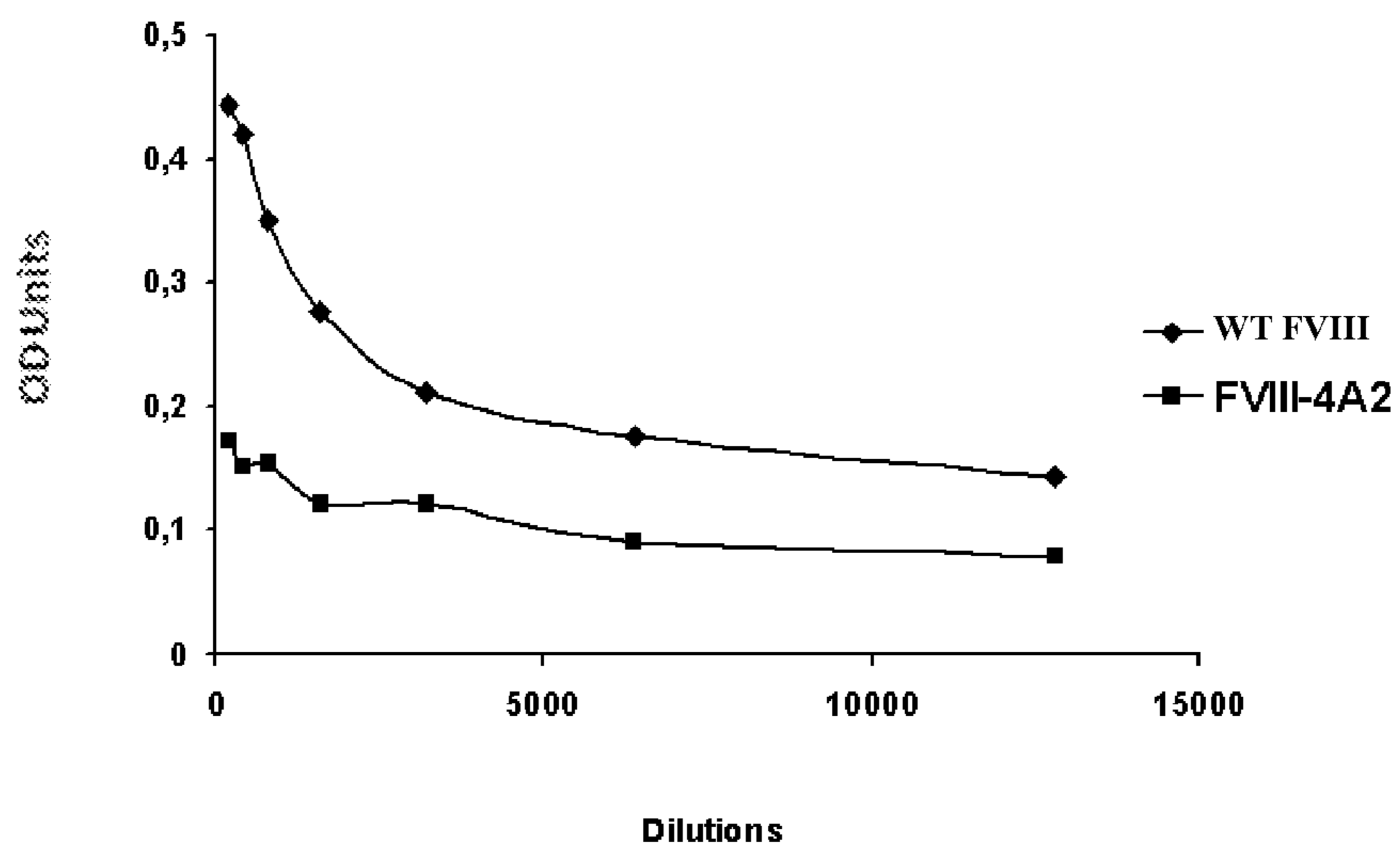


FIGURE 8A

### Titration of wild-type FVIII and FVIII-4A2 by ESH4 antibody

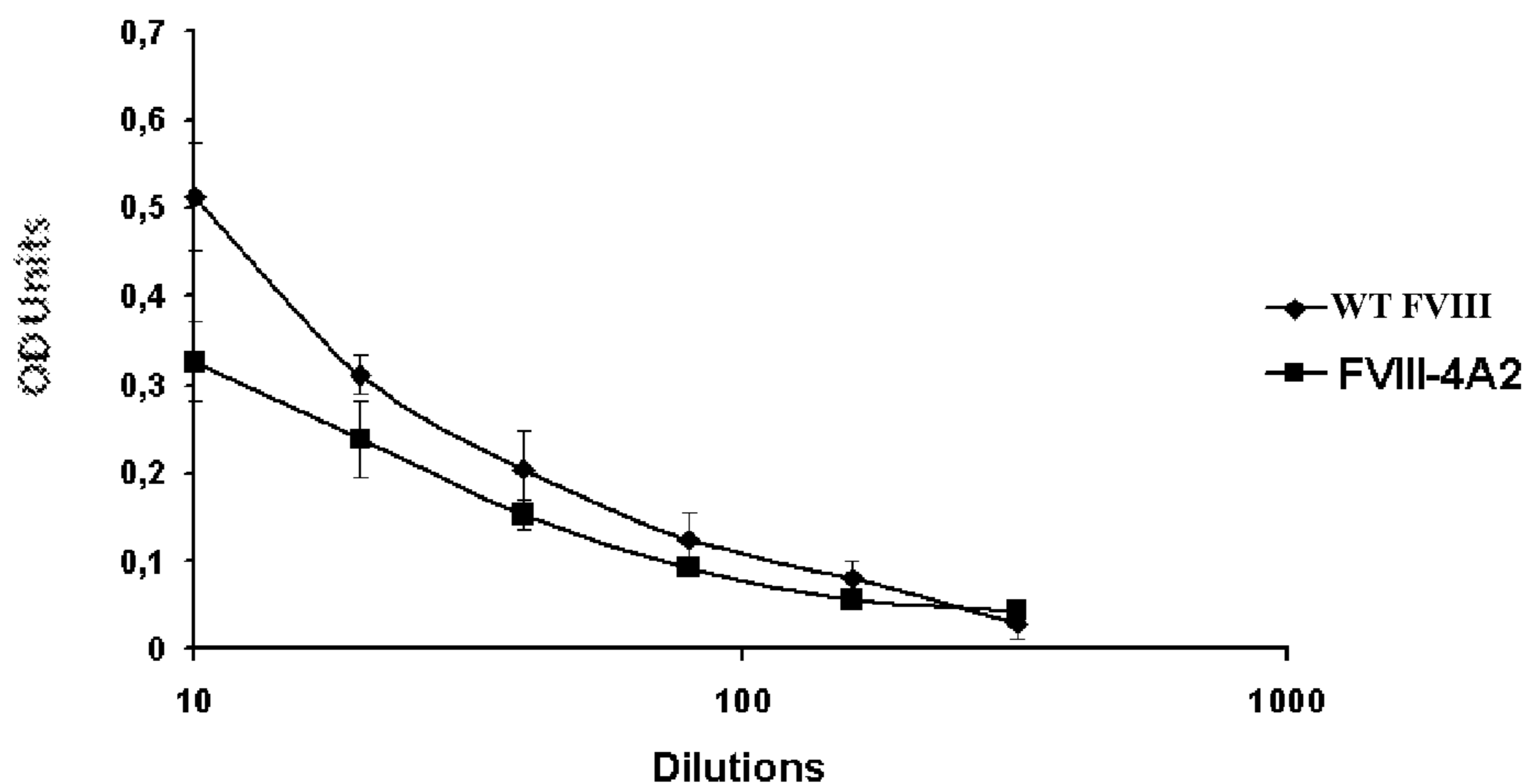


FIGURE 8B

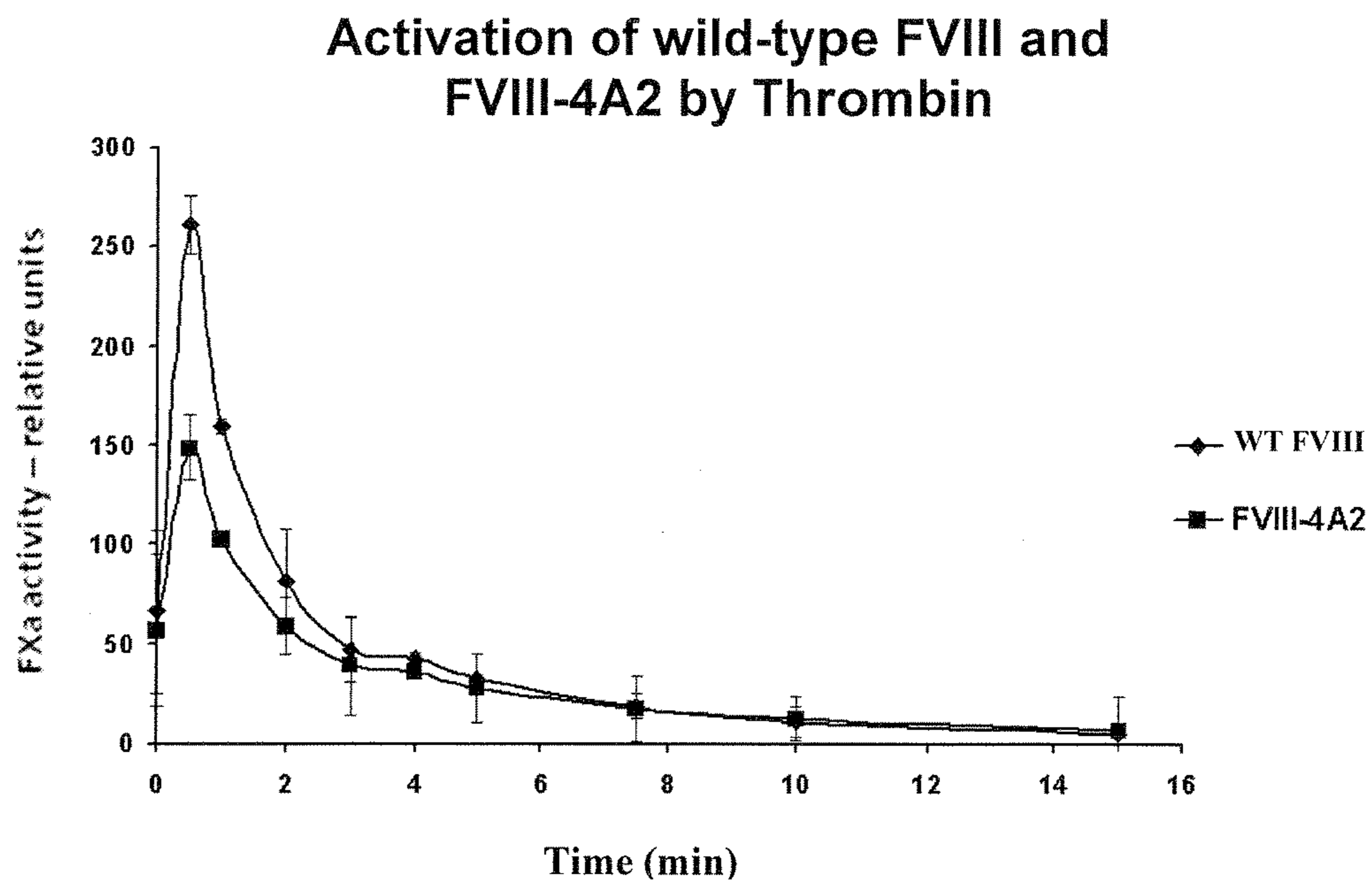


FIGURE 9



Loss of procoagulant activity after activation of wild-type FVIII and FVIII-4A2 by IIa

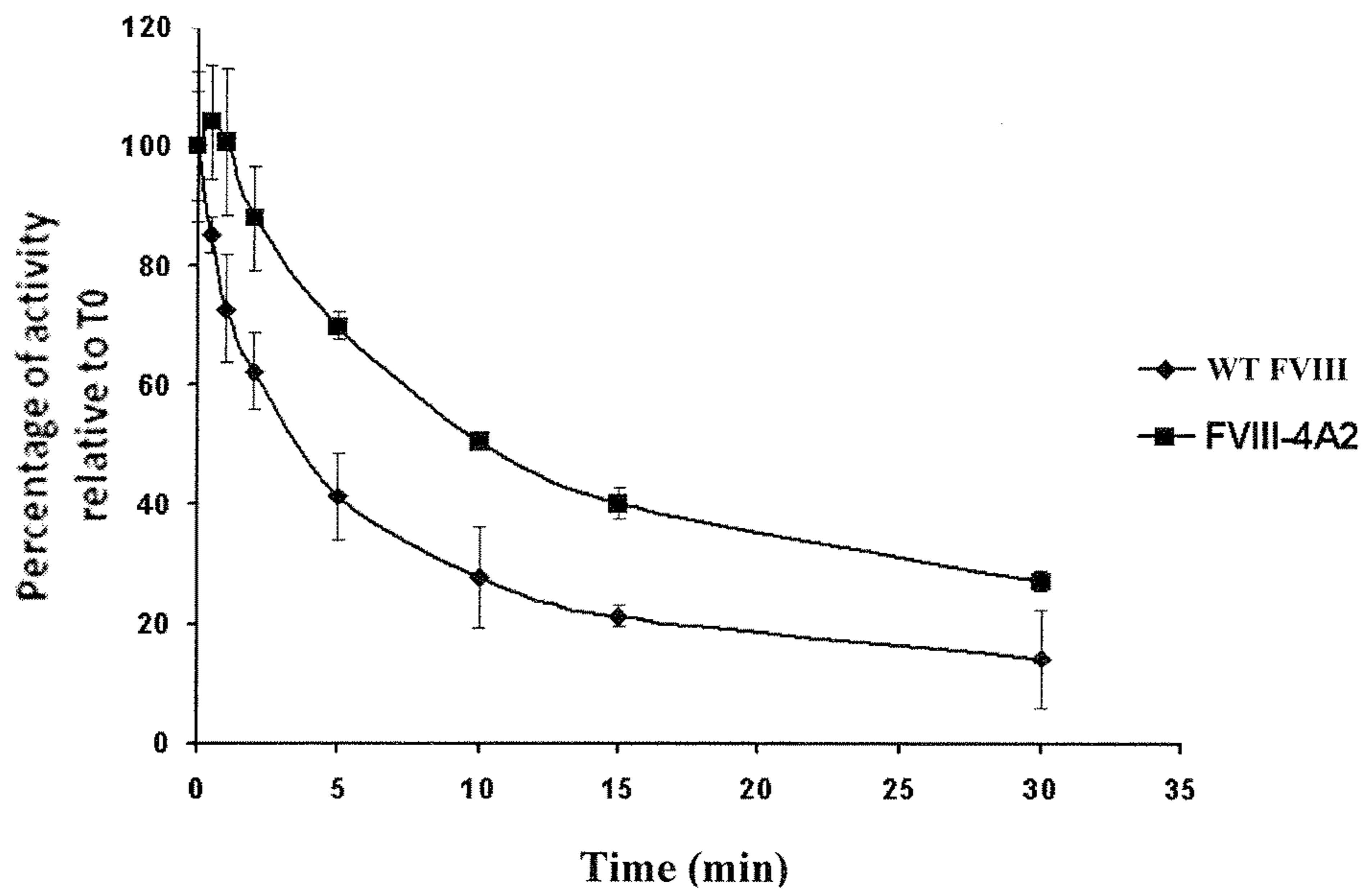
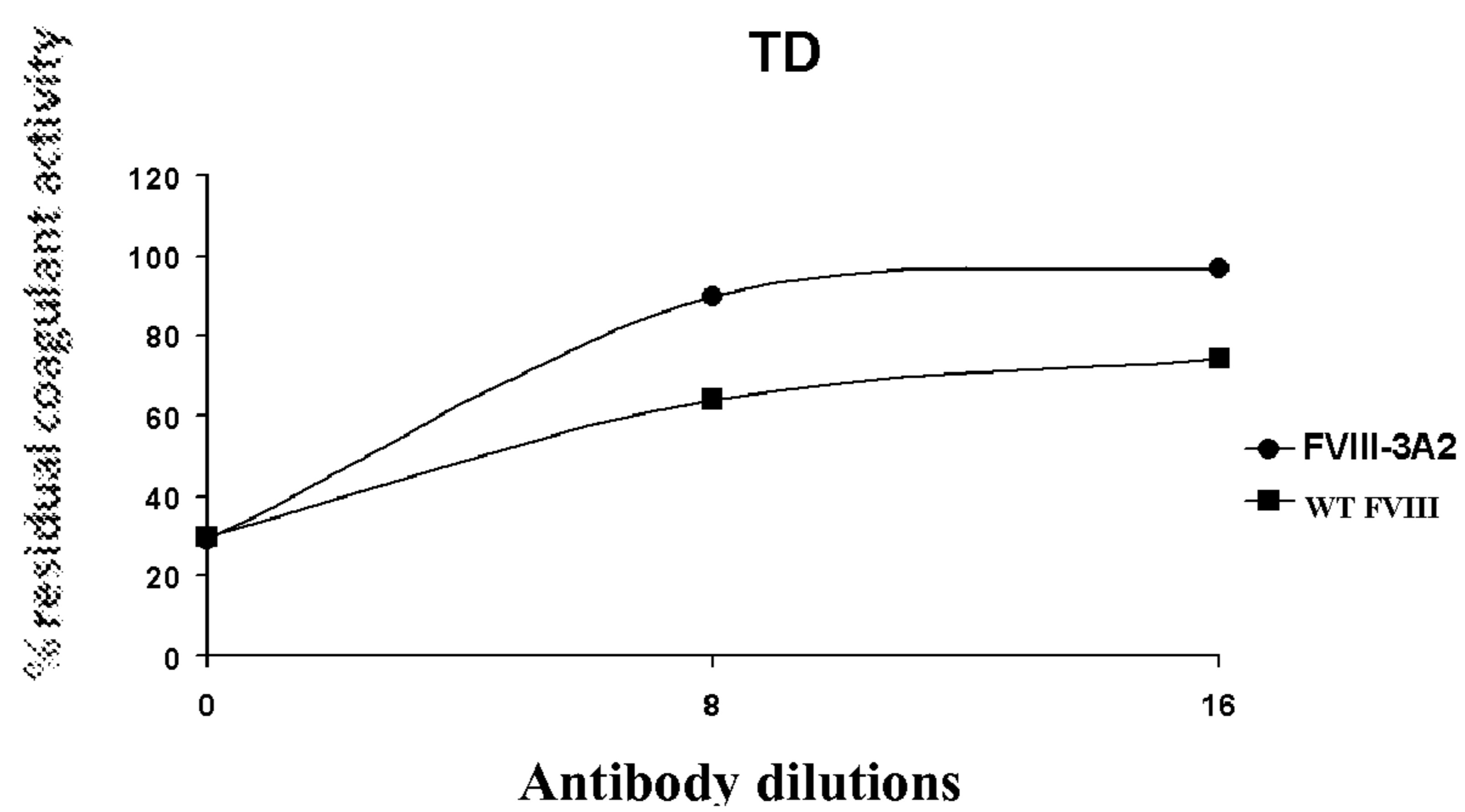
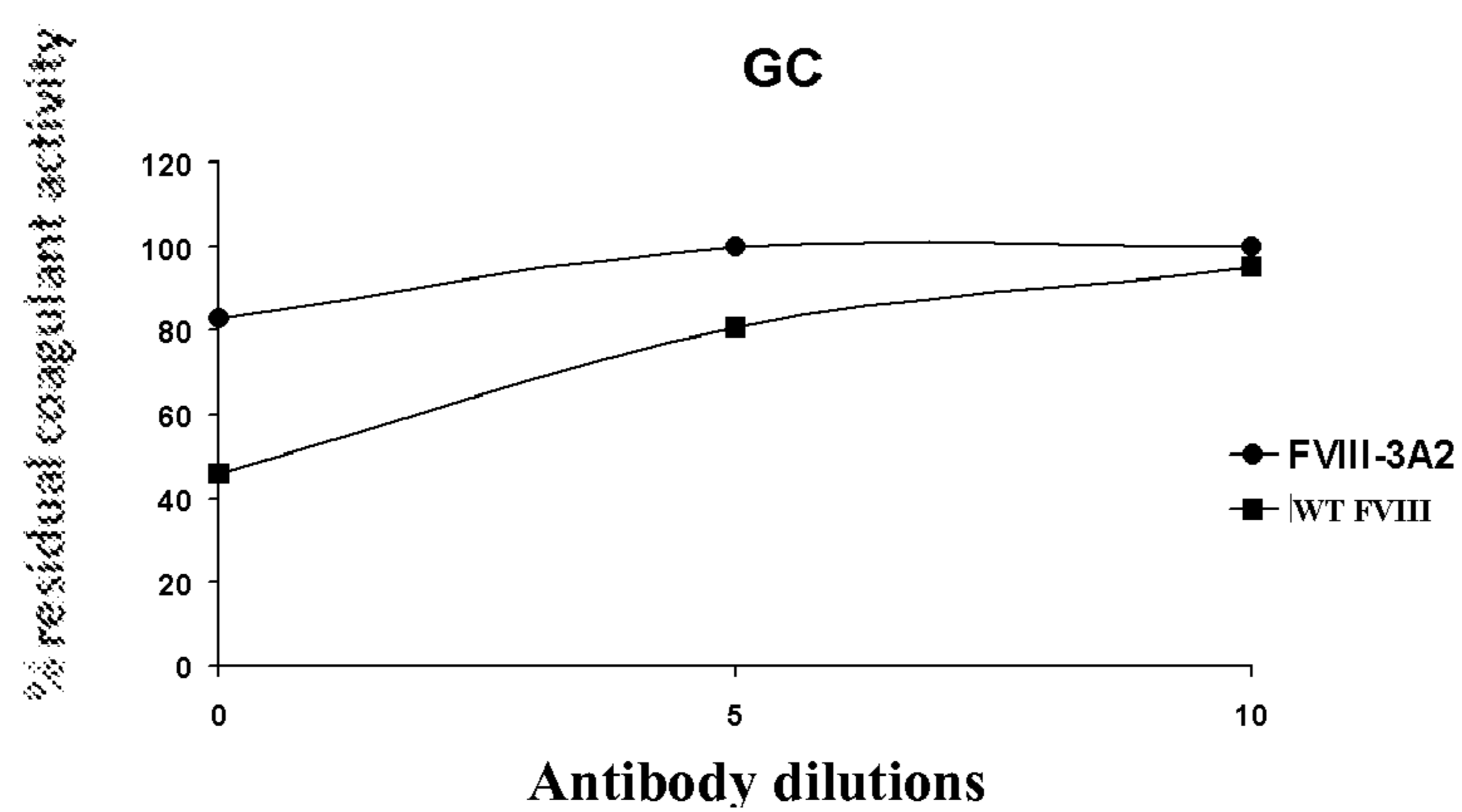


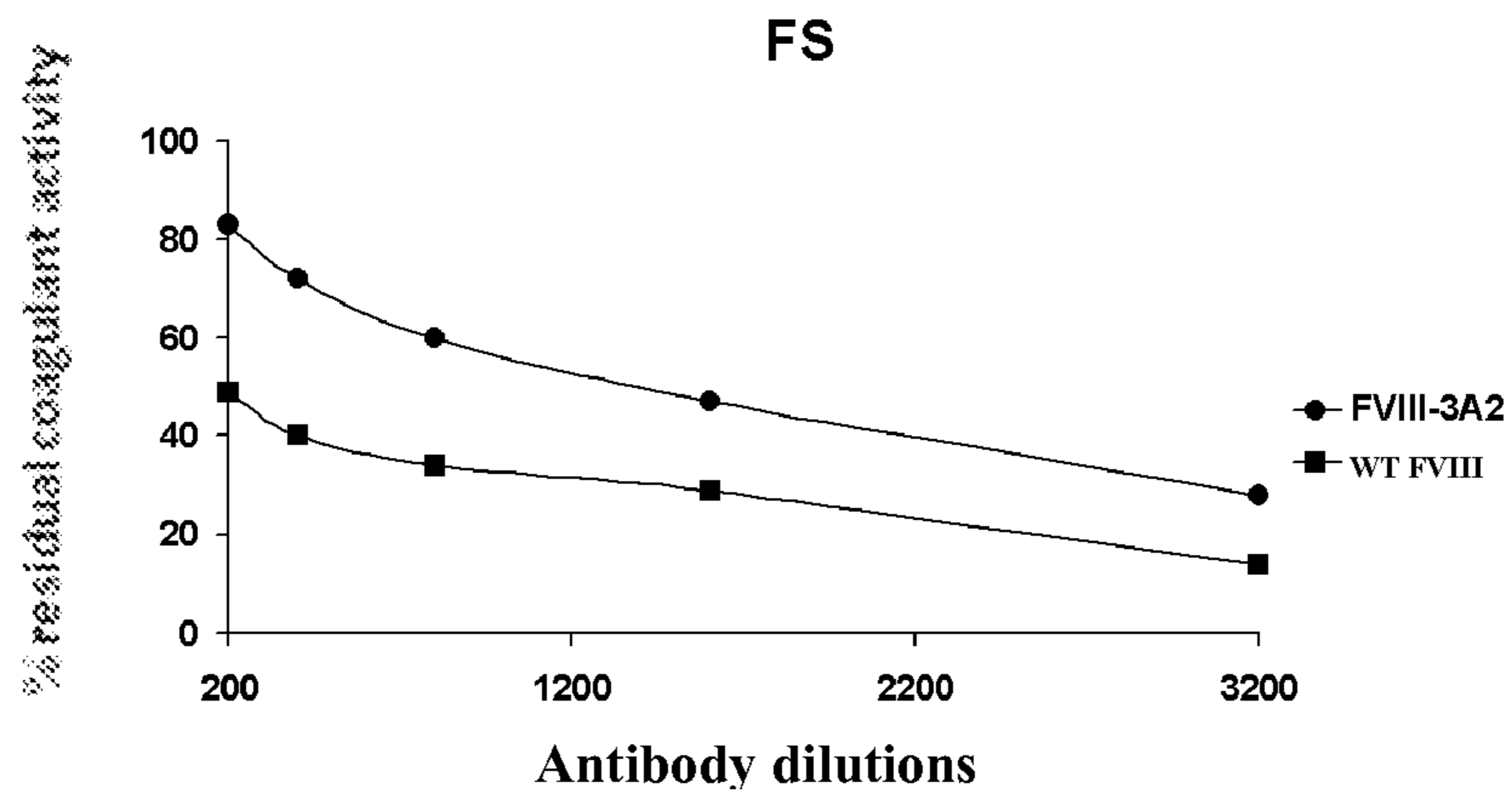
FIGURE 10



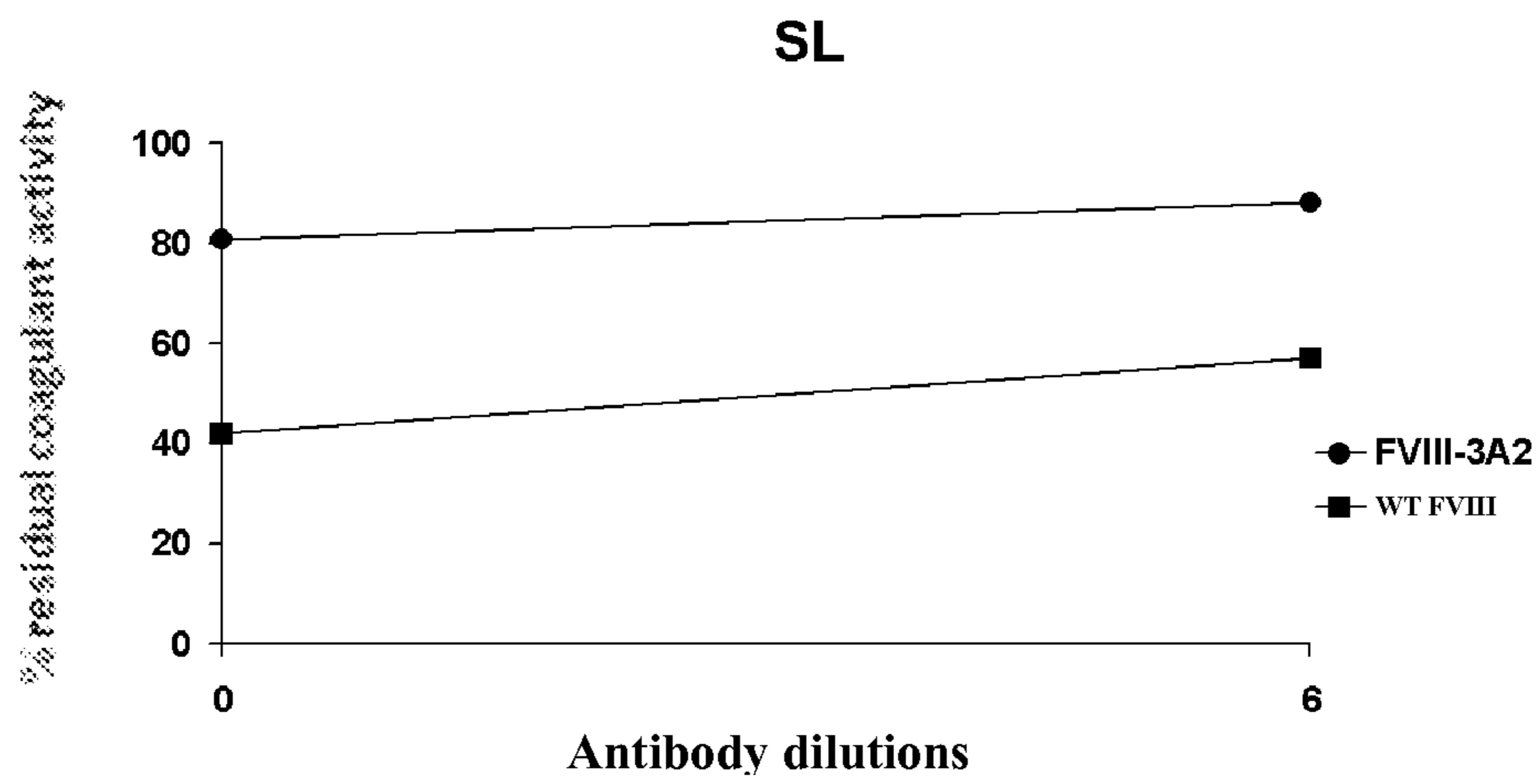
**FIGURE 11A**



**FIGURE 11B**



**FIGURE 11C**



**FIGURE 11D**

mutant	Activity (mUOD/min)	mutant	Activity (mUOD/min)	mutant	Activity (mUOD/min)	mutant	Activity (mUOD/min)
2175	103,5	2215	215	2269	98	2324	85
2177	125,5	2217	162,5	2270	123,5		
2181	81,6	2221	71,5	2273	126,5	WT	100
2182	85,3	2222	149,5	2274	88		
2183	120,5	2225	85,5	2275	81,5		
2186	80,5	2226	81	2277	151,5		
2189	74,8	2235	72,5	2278	133		
2191	175,6	2239	76	2280	116,5		
2195	86,8	2242	90,5	2281	88,2		
2196	152,5	2244	124,5	2282	75,5		
2197	71	2250	126,5	2284	114		
2199	114,5	2251	131	2289	162		
2200	131,4	2252	136,5	2292	136		
2202	75,3	2253	86,5	2294	113		
2204	92	2256	76	2296	52,8		
2205	78	2258	97,5	2311	137		
2206	105,5	2261	174,5	2312	129,5		
2212	134	2263	126,5	2316	162,5		
2213	141	2264	115	2317	64		
2214	121	2268	116,5	2321	58		

FIGURE 12

mutant	Activity (mUOD/min)	mutant	Activity (mUOD/min)	mutant	Activity (mUOD/min)	mutant	Activity (mUOD/min)
377	110,5	421	128	489	142,5	523	92
378	147,5	429	113	490	121,5	524	131,5
379	114,5	432	100,5	491	100,5	526	73
383	67	434	82	492	113,5	530	109
391	153	437	82	493	78,5	532	63
398	88,5	440	72,5	494	101	534	138,5
399	94,5	442	96	495	131,5	539	91
400	132,5	444	91,5	496	143	540	137,5
403	101,5	445	96	497	121	543	67,2
405	86	449	128,5	498	133	550	114
406	148	452	99	499	78	552	64,2
407	78,5	454	140	500	126	556	85
408	78	455	87	501	125,5	559	145
409	138	462	128,5	507	117,5	562	157
410	71	464	81	508	86	567	115,5
413	104,5	468	178	512	71,5	568	136,5
414	113,5	481	172,5	517	61,5	573	93
415	66,5	485	62	518	152,5	578	83
416	62	486	147	519	60	588	145
417	118	488	148,5	520	80,5	592	165

FIGURE 13

mutant	Activity (mUOD/min)
596	147,5
597	87
600	132
601	99,5
602	157,5
604	146,5
607	106
611	125,5
621	108,5
623	128,5
624	128,5
628	123,5
629	107,5
632	110
633	113
640	146
642	134,5

FIGURE 14

		<b>FS</b>	<b>TD</b>	<b>GC</b>	<b>PR</b>	<b>SL</b>
<b>Mutants</b>	<b>400</b>	<b>23</b>	<b>17</b>	<b>-</b>	<b>-</b>	<b>-</b>
	<b>486</b>	<b>14</b>	<b>24</b>	<b>10</b>	<b>14</b>	<b>-</b>
	<b>493</b>	<b>-</b>	<b>20</b>	<b>28</b>	<b>-</b>	<b>-</b>
	<b>403</b>	<b>34</b>	<b>-</b>	<b>10</b>	<b>-</b>	<b>16</b>
	<b>562</b>	<b>10</b>	<b>9</b>	<b>15</b>	<b>-</b>	<b>29</b>
	<b>414</b>	<b>33</b>	<b>9</b>	<b>-</b>	<b>-</b>	<b>-</b>
	<b>437</b>	<b>16</b>	<b>ND</b>	<b>-</b>	<b>-</b>	<b>-</b>

**FIGURE 15**

		<b>FS</b>	<b>TD</b>	<b>GC</b>	<b>PR</b>	<b>SL</b>
<b>Mutants</b>	<b>518</b>	-	22	-	-	-
	<b>2280</b>	-	34	12	6	21
	<b>2275</b>	-	-	10	19	24
	<b>2244</b>	-	38	-	-	25
	<b>2212</b>	25	16	-	-	-
	<b>2202</b>	-	18	-	-	-

**FIGURE 16**



		<b>FS</b>	<b>TD</b>	<b>GC</b>	<b>PR</b>	<b>SL</b>
<b>Mutants</b>	<b>421</b>	33	9	5	-	-
	<b>494</b>	-	17	28	5	-
	<b>496</b>	-	-	24	15	16
	<b>2206</b>	21	30	-	-	5
	<b>2226</b>	-	32	3	-	-
	<b>2261</b>	17	-	-	-	5
	<b>2281</b>	-	22	-	3	6
	<b>2282</b>	-	30	-	3	-
	<b>2311</b>	-	35	13	-	-

**FIGURE 17**

		FS	TD	GC	PR	SL
<b>Mutants</b>	<b>409</b>	31	12	5	-	15
	<b>462</b>	25	12	5	-	28
	<b>507</b>	-	27	5	5	-
	<b>629</b>	-	40	-	12	15
	<b>2312</b>	-	36	-	-	-
	<b>2289</b>	-	30	12	-	13
	<b>2316</b>	-	46	10	-	36
	<b>2294</b>	-	28	36	-	20

FIGURE 18

mutant	Activité muDO/min	Activité Spécifique muDO/min/ng/ml	mutant	Activité muDO/min	Activité Spécifique muDO/min/ng/ml
2202	75,3	7,52	400	132,5	13,6
2206	105,5	7,25	403	101,5	7,05
2212	134	6,89	409	138	12,35
2226	81	12,6	414	113,5	14,34
2244	124,5	10,02	421	128	19,21
2261	174,5	55,84	437	82	7,34
2275	81,5	11,54	462	128,5	16,8
2280	116,5	12,12	486	147	16,6
2281	88,2	17,37	493	78,5	18,06
2282	75,5	15,1	494	101	10,1
2289	162	8,67	496	143	15,03
2294	113	10,21	507	117,5	13,6
2311	137	24,4	518	152,5	11,5
2312	129,5	12,12	629	107,5	20,4
2316	162,5	10,67			
562	157	14,37	WT	100	3,2

FIGURE 19

409/462	409/2289	507/2312	2289/629
409/507	409/2316	507/2289	2312/2289
409/629	409/2294	507/2316	2312/2316
462/507	462/2312	507/2294	2312/2294
462/629	462/2289	2312/629	2289/2316
507/629	462/2316	2316/629	2289/2294
409/2312	462/2294	2294/629	2316/2294

**FIGURE 20**

	TD antibody Abolition to inhibition (%)	GC antibody Abolition to inhibition (%)	SL antibody Abolition to inhibition (%)	PR antibody Abolition to inhibition (%)	Specific activity (mUOD/ng/ml)
409/462	49	93	0	19	19,7
409/507	57	71	28	51	9,4
409/629	19	ND	ND	ND	6,14
462/507	57	57	6	49	12,1
462/629	33	93	94	43	7,1
507/629	12	12	0	10	7,04

FIGURE 21

## VIII FACTORS FOR THE TREATMENT OF TYPE A HEMOPHILIA

### CROSS-REFERENCE TO RELATED APPLICATION

This application is the U.S. national stage application of International Patent Application No. PCT/FR2008/050301, filed Feb. 22, 2008, the disclosure of which is hereby incorporated by reference in its entirety, including all figures, tables and amino acid or nucleic acid sequences.

### FIELD OF THE INVENTION

The present invention relates to the field of hemostasis, more specifically to that of hemophilia A. The invention relates to human factor VIII variants and to the uses thereof.

### TECHNICAL BACKGROUND

Factor VIII (FVIII) is mainly synthesized by hepatocytes and sinusoidal endothelial cells. The plasma concentration of FVIII is comprised between 0.1 and 0.2 mg/l; the circulating form is inactive and associates with von Willebrand factor (vWF). FVIII plays a key role in the endogenous (so-called intrinsic) pathway of blood coagulation. When a blood vessel is damaged by trauma, bleeding is triggered. In response, the process of hemostasis is initiated, consisting of a complex chain of events leading to the formation of a blood clot which seals the site of injury. Blood coagulation begins when platelets adhere to injured vessel walls. If the injury is severe, the platelet aggregates at the site of injury are insufficient to form a hemostatic plug to staunch the blood flow. Thus coagulation factors intervene whose purpose is to form the fibrin network, generated from soluble fibrinogen molecules by the action of thrombin. The formation of this network composed of insoluble fibers is crucial to firmly anchor the blood clot. Cascade shall be understood to mean that, sequentially and at each step, a precursor protein is converted to an activated protease which cleaves or acts as cofactor for cleavage of the next precursor protein of the cascade. Thus, FVIII is proteolytically cleaved in FVIIIa by the action of thrombin and factor Xa. In this active procoagulant form (FVIIIa), FVIII strikingly increases the proteolytic efficiency of factor FIXa towards factor FX.

Hemophilia A is a bleeding disorder characterized by a deficiency of activated FVIII due to a mutation in the recessive gene encoding FVIII. In some rare cases, hemophilia A may arise from the spontaneous development of auto-antibodies directed against FVIII; this is known as acquired hemophilia A.

Hemophilia is manifested as a defect of blood clotting in response to a hemorrhage. Untreated type A hemophiliacs exhibit symptoms such as excessive bleeding after trauma and sometimes even spontaneous hemorrhages, particularly into the articulation joints. Hemophilia A is the most common coagulation disorders and occurs in 1 in 5,000-10,000 male births. Not all hemophiliacs are affected in the same manner or to the same extent. For instance, hemophilia A is considered i) severe when FVIII levels are less than or equal to 1% of "normal" circulating levels; ii) moderate when FVIII levels are within the range of 1 to 5% of "normal"; and iii) mild when FVIII levels are between 5 and 30% of normal. These three types of hemophilia A occur at the following frequencies: 50% of hemophiliac patients have the severe form, 10% the moderate form and 40% the mild form.

Many genetic abnormalities have been associated with the gene coding for FVIII. Said gene is located at the tip of the long arm of the X chromosome (locus Xq28). Hemophilia A results from an abnormality in this gene. It is an X-linked recessive disorder: males and females can transmit the disorder but only males are affected. The molecular defects may be gene mutations, deletions or inversions. The majority of patients harboring missense point mutations have mild or moderate disease. Deletions are classified into two types: i) small deletions; ii) large deletions (>1 kb). Most large deletions confer a severe phenotype. With respect to genetic inversions, the intron 22 inversion is the most frequent and is responsible for the majority of cases of severe hemophilia A (45%). Another inversion, the intron 1 one, can cause severe disease while less frequent (3%).

In summary, these mutations result in either a decreased production of functionally normal FVIII molecules, or a quantitatively normal production of functionally defective FVIII molecules.

The FVIII gene codes for a polypeptide chain of 2,351 amino acids (aa) (SEQ ID No. 2) corresponding to a 19 aa signal peptide and a 2332 aa mature protein (330 kDa) (SEQ ID No. 3). The nucleotide sequence of the FVIII precursor is given in SEQ ID No. 1 and the corresponding protein sequence in SEQ ID No. 2. The FVIII precursor consists of a succession of the following seven functional domains: A1, a1, A2, a2, B, a3, A3, C1 and C2, from the N-terminal to the C-terminal (Vehar et al., 1984, Nature, 312:337-342).

FVIII undergoes a first intracellular proteolysis at arginines 1313 and 1648, producing a FVIII heterodimer consisting of: i) an A1-a1-A2-a2-B heavy chain; ii) an a3-A3-C1-C2 light chain. It circulates in plasma as a heterodimer. The interaction between the two chains is ensured among others by the presence of a chelated copper molecule in domains A1 and A3. Immediately after being secreted in plasma, FVIII forms a very high affinity association with von Willebrand factor (vWF) which protects it from proteases. FVIII and vWF form a noncovalent complex in which binding takes place mainly via two regions of FVIII: the N-terminal region and the C-terminal region at 2303-2332 (C2 domain) of the light chain. During coagulation, FVIII is cleaved by thrombin and factor Xa at three sites: i) thrombin cleaves at Arginine 1689 of the light chain and at Arginine 372 and Arginine 740 of the heavy chain; ii) factor XA cleaves FVIII at Arginine 336, Arginine 372 and Arginine 740. Two of these cleavages are common (Arginine 372 and Arginine 740). Cleavages at Arginine 372 and Arginine 1689 are essential for FVIII to participate in the coagulation cascade. These cleavages activate FVIII, also known as FVIIIa ("a" for "active"); in addition to FVIIIa activation, these cleavages result in removal of the 170 kDa B domain and dissociation of FVIIIa from vWF.

The B domain of FVIII, defined by amino acids 741 to 1648, can be totally or partially deleted with no loss of activity of recombinant FVIII (Toole et al., 1986, Proc. Natl. Acad. Sci. USA, 83 (16):5939-5942; Eaton et al., 1986, Biochemistry, 25 (26):8343-8347; Langer et al., 1988, Behring Inst. Mitt, 82:16-25; Meulien et al., 1988, Protein Eng, 2(4):301-6; and U.S. Pat. No. 4,868,112), including for porcine FVIII (U.S. Pat. No. 6,458,563; WO01/68109; U.S. Pat. No. 6,770,744), which in some cases can be used to replace the human FVIII.

Mutations, most of them point mutations, can be inserted at different sites of FVIII without causing a loss of FVIII procoagulant activity (U.S. Pat. Nos. 5,744,446; 5,859,204; 6,060,447; 6,180,371; 6,228,620; 6,376,463; EP 1561757;

WO02/24723; WO97/49725). EP1502921 and WO2005/111074 describe human FVIII variants with improved stability.

Other patents (US 2003/0083257; WO2005/040213; and U.S. Pat. No. 6,780,614) may be cited which describe modifications of FVIII cDNA for increasing its production in animal cells. The modifications of the cDNA are disclosed in patents US20021165177; US2002/0182684; EP1048726; EP1283263.

The number of units of FVIII administered is expressed in International Units (IU) with reference to the WHO standard for FVIII. FVIII activity is expressed either as a percentage (relative to normal human plasma) or in International Units (relative to an international standard). One International Unit (IU) of FVIII activity is equivalent to that quantity of FVIII contained in one milliliter of normal human plasma. Plas-  
matic FVIII assays may be carried out either by a chromo-  
metric method or by a chromogenic method.

Hemophilia A (severe and moderate forms) is generally treated by preventive or curative replacement therapy, which is based on repeated injections of the deficient coagulation factor or perfusion thereof. Patients with hemophilia A are treated with different types of plasma-derived or recombinant FVIII: i) recombinant; ii) semipurified plasma products; iii) plasma products purified on conventional or immunoaffinity columns. The first recombinant FVIII concentrates contained albumin as stabilizing agent. These included Kogenate® (Bayer), Helixate® (manufactured by Bayer, distributed by Aventis), and Recombinate® (Baxter). New albumin-free formulations have been developed, such as Kogenate® FS (Bayer), Helixate® FS (Bayer), and ReFacto<sup>MC</sup> (Wyeth). These nonetheless contain trace amounts of albumin arising from the cell culture medium used during the step of production of these recombinant proteins.

Recombinant human FVIII still needs to be optimized. Indeed, FVIII is relatively unstable in physiologic conditions, has a low activity in blood, is present at very low concentrations (0.1 to 0.2 µg/ml), and has a half-life of 10 to 12 hours.

In about 30% of severe hemophiliac A patients, replacement therapy causes complications specific to FVIII which lead to failure of the treatments usually used. In fact, after replacement therapy, patients may develop antibodies directed against the exogenous recombinant FVIII. These anti-FVIII antibodies inhibit the procoagulant activity of FVIII, hence the name “inhibitory antibodies” or else “inhibitors”. Further FVIII perfusion are rendered ineffective by these antibodies, and result in an increase of inhibitory antibody amount through a phenomenon known as “anamnestic reaction”.

Rapidly, patients can no longer be treated with FVIII, in which case the inhibitor “titer” is determined. This titer is expressed in international Bethesda units (BU). One BU of inhibitors corresponds to inactivation of half of the amount of FVIII in 1 ml of normal plasma. A titer is “low” when less than 10 BU, and “high” when more than 10 BU.

When the inhibitor titer is relatively low, hemophiliac patients may be given the aforementioned FVIII concentrates such as Kogenate® FS, Helixate® FS, Recombinate®, and ReFacto<sup>MC</sup>, but this carries a significant risk of inducing a rise in inhibitor titers which must therefore be closely monitored.

One of the ways to control inhibitory antibodies is to induce immune tolerance through administration of large doses of FVIII according to “de Bonn” protocol. In some patients, the inhibitory antibody titer is so high that they cannot be treated with large doses of FVIII for toxicity reasons.

A second approach known as the “Bonn-Malmo protocol” is based on one hand on ex vivo immunoabsorption of inhibitors immediately followed by reinjection of the blood, and on the other hand on injection of large doses of FVIII combined with immunosuppressive agents. These treatments are extremely costly in terms of recombinant FVIII and have achieved partial success.

Another approach consists in supplying coagulation factors in order to “bypass” the requirement of FVIII in the intrinsic coagulation pathway by using: i) plasma-derived activated prothrombin complex (FEIBA® VH, Factor Eight Inhibitor Bypassing Activity; Baxter) containing Factors II, VII, IX and X; ii) recombinant activated Factor VIIa (rFVIIa; NovoSeven®/Niastase®; NovoNordisk).

Said approaches have clear-cut success, nevertheless counterbalance by the development of side effects associated with this type of therapy (such as additional bleeding or conversely thrombotic events related to the frequency of administration).

It should be noted that circulating FVIII level increases after injection and then gradually declines related to its half-life. FVIII half-life ranges from 8 to 16 hours, with an average of 12 hours, raising the problem of repeated injections.

Another option consists in using a porcine FVIII with the aim to avoid antibodies directed against human FVIII. Patients who developed inhibitors to human FVIII have been successfully treated with semi-purified porcine FVIII (Hyate: C). Yet, this success has only been partial because after several injections of porcine FVIII, anti-porcine FVIII inhibitors have also developed, as mentioned in US2004/0249134. This phenomenon may necessitate to end treatment. Ipsen and Octagen are now co-developing a recombinant porcine FVIII known as OBI-1 in collaboration with Emory University in the USA, as a replacement for Hyate:C (WO2005107776).

Administration of porcine FVIII is therefore not a definitive solution for the treatment of hemophilia A patients with inhibitors.

As it can be seen, today there is no ideal treatment for individuals with hemophilia A, with or without inhibitors. The various problems encountered with commercial FVIII-based treatments associated with the development of these inhibitory antibodies have driven efforts to rapidly design a novel FVIII which has retained procoagulant specific activity and having lost the epitopes recognized by the inhibitory antibodies.

Few studies have addressed the epitope specificities of “inhibitory” antibodies. Some inhibitory antibodies appear to recognize small regions of the FVIII molecule: i) C2 domain in the light chain (2181-2321); ii) A2 domain in the heavy chain (484-509); iii) A3 domain (1694-2019) (Prescott et al., 1997, Blood, 89:3663-3671; Barrow et al., 2000, Blood, 95:557-561).

The 18 kDa C2 domain, between Serine 2173 and Tyrosine 2332, contains the membrane phospholipid binding domain and a part of the vWF binding domain. Inhibitory antibodies directed against the C2 domain mainly block the binding to phospholipids binding required for procoagulant activity but also the interaction with vWF. Mutations at positions Methionine 2199, Phenylalanine 2200, Valine 2223, Lysine 2227, Leucine 2251 and Leucine 2252 illustrate the importance of these amino acids in FVIII activity and binding to phospholipids and/or to vWF (Pratt et al., 1999, Nature, 402: 439-442).

Anti-A2 antibodies inhibit the function of FVIIIa as cofactor of Factor X (Lollar et al., 1994, J. Clin. Invest. 93:2497-2504). The main A2 epitope has been located between Arginine 484 and Leucine 508 (Healey et al., 1995, J. Biol. Chem., 270:14505-14509).

Antibodies directed against A3 and/or C2 domain prevent stabilization of the interaction between FVIII and vWF and also interfere with binding of the FVIII light chain to activated FIX.

Inhibitors are very heterogeneous from one patient to another and epitope specificity may change over time. Kinetic study of FVIII inhibition have revealed two types of allo-antibodies: type I antibodies which completely neutralize exogenous FVIII, and type II antibodies which never totally inhibit FVIII activity. Type II antibodies not completely block the procoagulant activity of FVIII because they are not saturable or display decreasing affinity according to their concentration.

Regions which can be recognized by inhibitory antibodies are cited in patents US2003/147900 and WO00/48635. These exposed and antigenic FVIII regions are between positions 1649-2019, 108-355, 403-725 and 2085-2249.

Moreover, US 2005/0256304 describes the following set of positions in human FVIII, where substitutions are likely to decrease antigenicity: 197, 198, 199, 201, 202, 407, 411, 412, 419, 515, 517, 613, 617, 636, 637, 638, 639, 823, 1011, 1013, 1208, 1209, 1210, 1254, 1255, 1257, 1262, 1264, 1268, 1119, 1120, 1121, 1122, and 1123.

The antigenicity of human FVIII can be decreased by glycosylation of recognition sites of inhibitors. Said method is disclosed in U.S. Pat. No. 6,759,216 and JP2004141173.

Another option consists in substituting the human FVIII epitopes usually recognized by inhibitors in domains: i) A2 (484-509); ii) A3 (1694-2019), a3 (1649-1687); iii) C2 (2181-2321). This solution is based on the use of a hybrid recombinant protein: a human/porcine FVIII.

The main targets of inhibitory antibodies are located in the A2 and C2 domains of FVIII (Saenko et al., Haemophilia, 2002). In fact, it is generally thought that 90% of inhibitory antibodies are directed against the human A2 and C2 domains (Barrow et al., 2000, Blood, 95:564-569). Moreover, it has been shown that human inhibitors have weak activity against porcine FVIII (Koshihara et al., Blood, 1995).

It is therefore expected that a substitution of human FVIII epitopes by porcine sequences would lead to a hybrid molecule less reactive towards inhibitory antibodies. Thus, the human A2 and C2 domains were replaced by their corresponding porcine domains (Lubin et al., 1994, J. Biol. Chem., 269:8639-8641). However, once again, anti-porcine FVIII antibodies eventually developed during the treatment of patients with inhibitors.

Many patents describe human/animal FVIII hybrids having retained a procoagulant activity. Human/animal hybrid, as used herein, denotes any combination (substitution) of at least one amino acid between a human FVIII sequence and a FVIII sequence of animal origin. Said hybrids have been produced, on the one hand, by substituting regions (functional subunits or structural domains) by the corresponding animal regions. For instance, U.S. Pat. Nos. 5,888,974; 5,663,060; 5,583,209; EP1359222; U.S. Pat. No. 5,744,446; WO93/20093; and WO95/24427 provide hybrid FVIII molecules derived from combinations of heavy and light chains of human and non-human FVIII, and/or derived from combinations of human/porcine FVIII domains.

U.S. Pat. No. 5,744,446 describes human/porcine FVIII variants wherein sequences of the human A2 domain are substituted by the corresponding murine or porcine sequences. The substituted fragments of the A2 domain are: 373-540; 373-508, 445-508, 484-508, 404-508, 489-508 and 484-489.

U.S. Pat. No. 5,364,771 provides a method for purifying FVIII hybrids derived from combinations of light and heavy

chains from human and non-human FVIII: human FVIII in which the A2 domain is replaced by the porcine A2 domain.

On the other hand, in some patents, said hybrids are formed by point substitutions of one or several amino acids of human FVIII by the corresponding amino acid(s) of animal origin (porcine, canine or murine). For example, US2004/0197875 discloses modifications in codon charges at certain positions of human FVIII. Said positions are defined related to porcine FVIII sequence. EP1454916 describes the introduction of porcine codons into the human cDNA.

Among these patents, studies have been addressed to develop human/porcine FVIII hybrids in the region of the A2 domain. EP1359222 describes a study of the porcine A2 domain sequence, with a view to generating such hybrid. US2003/166536; U.S. Pat. No. 6,376,463; WO00/71141 describe amino acid substitutions in human FVIII at key epitopes in the A2 domain, between positions 484 and 508: 486, 490, 491, 493, 494, 496, 498, 499, 500, 502, 503, 504, 505, 506, 507 for WO00/71141; and 485, 487, 488, 489, 492, 495, 501, 508 for U.S. Pat. No. 5,859,204. In particular, Alanine substitutions were made at positions: Arginine 484, Proline 485, Tyrosine 487, Serine 488, Arginine 489, Proline 492, Valine 495, Phenylalanine 501, and Isoleucine 508. These substitutions conferring decreased antigenicity might

be of interest from a therapeutic standpoint.

Likewise, in U.S. Pat. No. 6,180,371, Arginine 484 is substituted by Serine, Proline 485 by Alanine, Arginine 489 by Glycine, Proline 492 by Leucine. With these variants, inhibition of the procoagulant function of FVIII by antibodies was alleviated or disappeared altogether. The therapeutic interest of a double or triple mutant at Arginine 484, Arginine 489 and Phenylalanine 501, where each codon is substituted with an Alanine, is suggested.

There are also patents disclosing FVIII variants in which the substitutions only affect the C2 domain.

US2004/249134; WO03/047507; WO02/24723; U.S. Pat. No. 6,770,744 describe substitutions at positions Methionine 2199, Phenylalanine 2200, Valine 2223, Lysine 2227, Leucine 2251 and Leucine 2252. Said substitutions were introduced into a FVIII lacking the B domain. Amino acids at positions 2215, 2220, 2320, 2195, 2196, 2290 and 2313 were substituted with an Alanine.

With regard to position 2223, Valine is replaced by an Alanine, by comparison between human and porcine FVIII. This mutation is mentioned in Pratt's article "Structure of the C2 domain of human FVIII" (Nature, 1999, 402:439-442) and in U.S. Pat. No. 6,770,744.

Combinations of certain mutated positions such as 2199, 2200, 2223 and 2227 have been described as reducing the antigenicity of FVIII with regard to some anti-C2 domain inhibitory antibodies, all while retaining the coagulant activity of FVIII.

In patents WO99/46274 and US2005/0079584, J. Lollar's group describes a region of potential interest for constituting a less immunogenic FVIII: 2181 to 2243. This region was defined very roughly by an antigenicity study of human/porcine hybrids. An alignment between human and porcine FVIII of the sequence 2181 to 2243 disclosed 17 differences at the following positions: 2181, 2182, 2195, 2196, 2197, 2199, 2207, 2216, 2222, 2224, 2225, 2226, 2227, 2228, 2234, 2238, 2243. J. Lollar's group speculate that a substitution at these 17 positions by an Alanine, a Methionine, a Serine, a Glycine, or else a Leucine might generate a FVIII protein that can avoid inhibitory antibodies. This hypothesis is not supported by any antigenicity studies of mutants of interest.

Lastly, patents such as U.S. Pat. No. 6,180,371; US2002/182670; US 2003/068785; US2005/079584; WO99/46274;



U.S. Pat. No. 7,012,132; WO2005/046583 provide human/porcine hybrids harboring substitutions in both the A2 and C2 domains of FVIII with the aim of reducing inhibition by inhibitory antibodies that recognize both domains. In particular, WO2005/046583 describes amino acid substitutions in the A2 and C2 domains at positions 484, 489, 492, 2199, 2200, 2251 and 2252. The FVIII which was used lacks the B domain. Only position 484 has an Arginine substituted by an Alanine.

To summarize, while many studies make reference to novel FVIII variants, there is still a need for a novel, less immunogenic FVIII, because there are no modified FVIII variants capable of treating patients with inhibitors currently on the market. Moreover, variants with an improved specific activity or an improved capacity to be secreted are also of major interest to promote the production of recombinant FVIII or to improve the treatment of patients.

#### SUMMARY OF THE INVENTION

The present invention therefore provides novel improved FVIII variants. Said variants may have lost the epitopes recognized by inhibitory antibodies all while retaining the core of their procoagulant activity, or have an improved specific activity, or else have an improved secretion capacity. Said variants may also have a combination of these features. For example, the invention relates to variants which are less immunogenic and have an improved specific activity and/or an improved secretion capacity. Likewise, the invention relates to variants having an improved specific activity and/or an improved secretion capacity.

A first object of the present invention is an improved human FVIII variant or a biologically active derivative thereof comprising a substitution of at least one amino acid selected from the group consisting of the residues at positions 462, 409, 507, 629, 400, 562, 403, 518, 414, 496, 421, 493, 486, and 494 of the A2 domain and the residues at positions 2206, 2212, 2226, 2244, 2261, 2275, 2280, 2281, 2282, 2289, 2294, 2311, 2312, and 2316 of the C2 domain. In a particular embodiment, the human FVIII variant or biologically active derivative thereof consists of a single substitution. In another particular embodiment, the human FVIII variant or biologically active derivative thereof further comprises a substitution of at least one amino acid selected from the group consisting of the residue at position 2202 of the C2 domain and the residue at position 437 of the A2 domain. In a particular embodiment, the human FVIII variant or biologically active derivative thereof comprises the substitution of at least two, three, four, five six, seven, eight, nine, ten, eleven, twelve, thirteen, fourteen or fifteen amino acids, preferably selected from the aforementioned groups. Preferably, the amino acid is substituted by an amino acid selected from an Alanine, a Methionine, a Serine, a Glycine, and a Leucine. More preferably, the amino acid is substituted by an Alanine. Preferably, the biologically active FVIII derivative is a FVIII consisting in a partial or whole deletion of the B domain.

In a particular embodiment, the variant has decreased antigenicity towards inhibitory antibodies as compared to natural human FVIII and retains a procoagulant activity at least equal to 50% of that of natural human FVIII. In a preferred embodiment, the invention relates to an improved human FVIII variant or a biologically active derivative thereof comprising a substitution of at least one amino acid selected from the group consisting of the residues at positions 462, 409, 507 and 629 of the A2 domain and the residues at positions 2289, 2294, 2312, and 2316 of the C2 domain. Said variant can further comprise a substitution of at least one amino acid selected

from the group consisting of the residue at position 2202 of the C2 domain and the residue at position 437 of the A2 domain. In a more preferred embodiment, the invention relates to an improved human FVIII variant or a biologically active derivative thereof comprising the substitution of at least one amino acid selected from the group consisting of the residues at positions 462, 409, 507 and 629 of the A2 domain. In another embodiment, the invention relates to an improved human FVIII variant or a biologically active derivative thereof comprising or consisting of the combination of two substitutions selected from the group consisting of 409+462, 409+507, 462+507, 409+629, 462+629, 507+629. In yet another embodiment, the invention relates to an improved human FVIII variant or a biologically active derivative thereof comprising or consisting of the combination of three substitutions selected from the group consisting of 409+462+507, 462+507+629, 409+462+629, 409+507+629. In another particular embodiment, the invention relates to an improved human FVIII variant or biologically active derivative thereof comprising or consisting of the combination of four substitutions at positions 409, 462, 507 and 629.

Furthermore, these mutations which confer abolition to inhibition by inhibitory antibodies may prove to be of great interest in combination with mutations conferring a higher specific activity, allowing compensating an optional relative loss of activity of these less antigenic mutants. In a particular embodiment, the variant has an improved specific activity as compared to that of natural human FVIII. In a preferred embodiment, the invention relates to an improved human FVIII variant or a biologically active derivative thereof further comprising the substitution of at least one amino acid selected from the group consisting of the residues at positions 2177, 2183, 2186, 2191, 2196, 2204, 2205, 2213, 2217, 2235, 2258, 2264, 2268 and 2269 of the C2 domain.

Said mutations which confer abolition to inhibition by inhibitory antibodies may also prove to be of great interest in combination with mutations conferring an improved capacity to be secreted, by allowing compensating an optional relative loss of secretion of these less antigenic mutants. In a particular embodiment, the invention relates to an improved human FVIII variant or a biologically active derivative thereof further comprising the substitution of at least one amino acid selected from the group consisting of the residues at positions 2175, 2199, 2200, 2215, 2251, 2252 and 2278 of the C2 domain. Massive production of mutants having retained at least 50% of FVIII activity also makes it possible to encompass their use in a context of analyzing additional functions of the protein. In addition to a modulation of its immunogenicity, secretion and specific activity, the following properties of FVIII might be improved by using the herein described mutated molecules: —binding to von Willebrand factor and therefore improved half-life of FVIII or circulating FVIIIa; —improved intrinsic stability of the molecule by stabilization of the A2 domain and therefore an increased efficiency period; —binding to phospholipids derived from blood platelets, cell surfaces or circulating microparticles and therefore improved formation of FXa; —binding to FIXa and FX and therefore improved formation of FXa; —decreased binding of FVIII or FVIIIa to the molecules responsible for its catabolism such as for example low density Lipoprotein Receptor-related Protein (LRP), Low density Lipoprotein Receptor (LDLR), Very Low Density Lipoprotein Receptor (VLDLR), megalin or any other receptor which might be identified and therefore improved half-life of circulating FVIII; —proteolysis decrease of FVIII or FVIIIa by vascular proteases such as for example activated protein C, FXa, FIXa, and therefore increase efficiency period.

A second object of the present invention relates to a nucleic acid coding for a human FVIII variant or a biologically active derivative thereof according to the invention, an expression cassette comprising said nucleic acid, a vector, preferably an expression vector, comprising said nucleic acid or said expression cassette, and a host cell comprising a nucleic acid, an expression cassette or a vector according to the present invention. Preferably, the vector can be selected from a plasmid and a viral vector. The present invention also relates to the use of a nucleic acid, an expression cassette, an expression vector or a host cell according to the invention for producing a human FVIII variant or a biologically active derivative thereof according to the present invention.

A third object of the present invention relates to a pharmaceutical composition comprising a human FVIII variant or a biologically active derivative thereof according to the invention. Thus, the present invention relates to a human FVIII variant or a biologically active derivative thereof according to the invention as medicament. The present invention further relates to a human FVIII variant or a biologically active derivative thereof according to the invention for the treatment of hemophilia A. The treatment can be curative or preventive. In a particular embodiment, the patient to be treated is a patient with inhibitors. In another embodiment, the patient to be treated is a hemophiliac patient before any development of inhibitors. The present invention equally relates to a method for treating hemophilia A comprising administering a human FVIII variant or a biologically active derivative thereof according to the present invention.

A fourth object of the present invention relates to the use of a human FVIII variant or a biologically active derivative thereof according to the invention for preparing a medicament for the treatment of hemophilia A. The treatment can be curative or preventive. In a particular embodiment, the patient to be treated is a patient with inhibitors. In another embodiment, the patient to be treated is a hemophiliac patient before development of any optional inhibitors. The present invention also relates to a method for treating hemophilia A comprising administering a human FVIII variant or a biologically active derivative thereof according to the present invention.

A fifth object of the present invention relates to the use of one or more human FVIII variants or a biologically active derivative thereof according to the present invention for the diagnosis of inhibitor type in a patient with hemophilia A.

#### BRIEF DESCRIPTION OF FIGURES AND TABLES

FIG. 1: Simplified scheme of the coagulation cascade. Ca: calcium-dependent step. PL: phospholipids of blood platelet membrane. TF: tissue factor. TFPI: tissue factor pathway inhibitor. The role of FVIIIa is to increase the catalytic efficiency of FIXa to activate FX. Assembly of FXa and FVa triggers a significant increase in thrombin formation.

FIG. 2A-2E: Primary screen results: Raw activities of 359 Alanine mutants over the 795 produced=functional mapping of FVIII activity of these 359 positions.

FIG. 3: Production of FVIII in culture medium; 8 mutants displayed a much higher production level than non-mutated FVIII in the same conditions.

FIG. 4: Highest specific activities of 15 mutants compared to non-mutated FVIII in the same conditions.

FIG. 5: Example of determining abolition of the serum TD to inhibition by FVIII mutant E518A. Abolition to inhibition is expressed as a percentage:  $[(b-a)/a] \times 100$ ; where "a" represents residual activity percentage of the WT (serum+IgG/

serum-IgG) and "b" is the residual activity percentage of the mutant (serum+IgG/serum-IgG).

FIG. 6A-6E: Abolition of FVIII-4A2 versus wild-type FVIII to inhibition by inhibitory antibodies from five patients (TD, GC, PR, SL and FS) measured by Bethesda assay.

Residual activity, determined after incubation with inhibitory antibodies, is divided by remained activity after incubation with a non-immune antibody; the residual activity percentage is thus determined.

FIG. 7A-7B: Determining the inhibition decrease of the FVIII-4A2 mutant by anti-A2 domain antibody (GMA012) and a rabbit polyclonal antibody.

FIG. 8A-8B: Comparative titration on a solid support of FVIII-4A2 versus wild-type FVIII by ELISA using anti-C2 domain antibody (ESH4) and anti-A2 domain antibody (GMA012).

FIG. 9: Comparative determination of FVIII-4A2 and wild-type FVIII activation by thrombin.

FIG. 10: Comparative determination of A2 domain dissociation and resultant loss of activity for FVIII-4A2 and wild-type FVIII after activation by thrombin (IIa).

FIG. 11A-11D: Abolition of FVIII-3A2 versus wild-type FVIII to inhibition by inhibitory antibodies from four patients (TD, GC, SL and FS) measured by Bethesda assay.

FIGS. 12-14: Primary screen results; list of 158 Alanine mutants selected for secondary screening, having retained at least 50% of raw activity relative to non-mutated FVIII activity.

FIGS. 15-18: Secondary screening: Bethesda assays on 30 mutants displaying modified antigenicity towards sera from five hemophiliac patients with inhibitors. Results are expressed as the abolition to inhibition percentage for each mutant as exemplified in FIG. 5.

FIG. 19: Comparison of specific activity and raw activity relative to non-mutated FVIII activity for the 30 mutants displaying modified antigenicity towards sera from five hemophiliac patients with inhibitors.

FIG. 20: List of all FVIII double mutants produced from the eight single mutants FVIII409A, FVIII462A, FVIII507A, FVIII629A, FVIII2289A, FVIII2294A, FVIII2312A and FVIII2316A.

FIG. 21: Chromogenic specific activities and abolition to inhibition percentages towards inhibitory antibodies of six double A2 mutants from sera of four hemophiliac patients TD, GC, SL and PR.

#### Description of the invention

The present invention provides a solution to resolve a serious complication that occurs in 30% of hemophilia A patients treated with recombinant FVIII: the development of an immune response induced by the treatment and directed against the exogenous recombinant FVIII. The solution provided consists in generating recombinant human FVIII molecules having decreased antigenicity of the epitopes usually recognized by inhibitory antibodies. The FVIII variants of the invention have lost one or more epitopes usually recognized by said antibodies.

The present invention provides other solutions consisting in generating human FVIII variants having an improved specific activity as compared to natural FVIII.

Lastly, the present invention provides with FVIII variants having a greater capacity to be secreted, which is interesting for the production of recombinant FVIII and in a potential gene therapy.

The different properties conferred by the mutations in these variants may be of major interest in combination. In a non-limiting example, mutations which confer a specific activity improvement of a variant could compensate an

optional relative loss of activity in variants whose mutations confer a abolition to inhibition by inhibitory antibodies and being therefore less antigenic. In another non-limiting example, mutations which confer a higher capacity to be secreted may interesting in combination with mutations conferring an abolition to inhibition by inhibitory antibodies, by allowing, for example, to compensate a optional relative loss of secretion of said less antigenic mutants.

In the present document, the following terminology is used to designate a substitution: **5409A** indicates the substitution of the serine residue at position **409** of SEQ ID No. **3** by an alanine. Substitution refers to the replacement of an amino acid residue by another one selected from the other 19 amino acids or by a non-naturally occurring amino acid. The terms “substitution” and “mutation” are interchangeable. The sign “+” indicates a combination of substitutions.

“Comprise” means that the variant or the fragment thereof has one or more substitutions such as indicated with reference to SEQ ID No. **3**, but that the variant or the fragment thereof may have other modifications, particularly substitutions, deletions or insertions.

the chromogenic assay mentioned above. This assay was also performed on the robotic platform of the National Hemophilia Treatment Center (Hospices Civils de Lyon). The chromogenic activity of the **158** selected Alanine mutants was carried out with the Coamatic Factor VIII kit (Chromogenix, Instrumentation Laboratory, Milan, Italy) according to the supplier’s instructions. Briefly, culture supernatants (50  $\mu$ l) were diluted in the dilution buffer provided and preincubated at 37° C. for 4 min. The reaction medium (50  $\mu$ l), preheated at 37° C., was then added for 4 min, after which 50  $\mu$ l of development medium at 37° C. were added. The formation of product over time was measured immediately on a spectrophotometer at 405 nm after shaking the microtiter plate. Product formation is expressed as mUOD/min. When values were greater than 200 mUOD/min, the assay was repeated using a higher dilution.

FIGS. **12-14** show the activities of the **158** mutants which retained more than 50% of non-mutated FVIII activity. Said **158** mutants were selected for the secondary screening.

Example 4: Secondary screen: Evaluation of loss of antigenicity towards human FVIII inhibitory antibodies

The secondary screen correlates to an assay similar to the Bethesda assay, carried out as described below on the **158** mutants selected following the primary screening; said assay comprises a step of contacting a inhibitory serum (or antibody) with a FVIII molecule to be tested or a reference standard and a step of measuring FVIII coagulant activity by chromometric assay.

Culture supernatants obtained after **48** h of contact with COS cells transfected by different FVIII constructs were used. Said supernatants were produced in complete medium [(IMDM, Invitrogen), 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin]. Supernatants were diluted in fresh complete medium to obtain a final chromometric activity comprised in the range of about 10-20% (1 FVIII unit = 100% activity = 200 ng/ml). The culture supernatant diluted or not (140  $\mu$ l) was added to 150  $\mu$ l of FVIII-depleted human plasma (Stago, Asnieres, France). An antibody dilution (10  $\mu$ l) was then added to the mix. These antibodies are IgG fractions purified on protein A- from hemophiliac patients with inhibitors. An IgG fraction from a non-hemophiliac control was similarly obtained. Bethesda inhibitor titers were identical to the inhibitory activity from the plasma. The purification protocol therefore did not affect the inhibitory activity of the antibodies. The antibodies were first diluted in fresh complete medium, the measurement

being carried out either with a fixed antibody dilution or with serial dilutions. The fixed antibody concentration which was used was that which produced 50% inhibition of a recombinant FVIII standard solution with 12.5% activity. Samples were incubated in a 37° C. water-bath for 1h30. Coagulant activity was then determined on a MDA-II apparatus (BioMérieux, Marcy-l’Etoile) and compared to that of a standard curve established from an identical FVIII stably produced in the CHO cell line. Results are expressed as a percentage which represents the abolition to inhibition of coagulant activity of a given mutant by inhibitory antibodies from a patient’s serum. Said percentage was calculated as shown in FIG. **5** for the FVIII mutant **E518A**. Abolition to inhibition expressed is a percentage =  $-(b-a)/a \times 100$ ; where “a” is the percentage residual activity of the WT (serum + IgG / serum - IgG) and “b” is the percentage residual activity of the mutant (serum + IgG/serum - IgG).

FIGS. **15-18** show for 30 single mutants the percentages of abolition to inhibition for sera from five hemophiliac patients. Said mutants were selected in the secondary screen of the 158 mutants selected in the primary screen. Several mutants show a high percentage of abolition to inhibition with certain sera, such as mutant **2316** for sera TD and SL, mutant **2294** for serum GC, mutant **403** for serum FS and mutant **2275** for serum PR.

Patients’ sera were selected for their high Bethesda titers (greater than 10 BU) and their different inhibitor profiles. These patients can no longer be treated with FVIII injections and need bypassing agents. Thus, obtaining FVIII Alanine mutants which abolish, even partially, the inhibition of FVIII activity by the inhibitory antibodies of one of these patients, is a major step forward to the future approaches of treating hemophiliac patients with inhibitors. The different data obtained on a large number of mutants as well as the different sera tested will make it possible to create combinations of mutations leading to an improved FVIII which can avoid a majority of inhibitory antibodies while retaining its procoagulant activity.

The reproducibility of FVIII expression level related to transfections was controlled by following the specific activity of wild-type FVIII. Indeed, specific activities calculated from antigen determinations (Stago commercial ELISA kit) were identical for wild-type FVIII produced in different transfections. Likewise, antigen concentrations were determined for mutants having retained at least 50% of wild-type FVIII activity and their specific activity was determinate throw. Specific activity corresponds to raw activity measured in the chromogenic assay (mUOD/min) relative to protein concentration (ng/ml) obtained with an ELISA kit (Stago FVIII kit). FIG. **19** shows comparative data of raw and specific activities of 30 mutants selected in the secondary screen.

The eight FVIII Alanine mutants **2175**, **2199**, **2200**, **2215**, **2251**, **2252**, **2278** and **2316** displayed a far above average capacity to be secreted in the COS cell production medium used in the scope of the present invention. FIG. **3** depicts the data obtained for these eight mutants. Raw coagulant activity of these mutants was determined by chromogenic assay. Their concentration was approximately two to four times higher than that of wild-type FVIII. This property is interesting for producing recombinant FVIII and might make it possible to lower production costs of a new generation FVIII. Also, it might be advantageous in a gene therapy for hemophiliac patients. Moreover, these mutations which confer a greater capacity to be secreted may be of major interest in combination with mutations conferring abolition to inhibi-

tion by inhibitory antibodies, by allowing, for example, to compensate an optional relative loss of secretion of said less antigenic mutants.

The 15 mutants **2177, 2183, 2186, 2191, 2196, 2204, 2205, 2206, 2213, 2217, 2235, 2258, 2264, 2268** and **2269** displayed far higher specific activity than wild-type FVIII, while maintaining a high production level, around to that of wild-type FVIII (concentration greater than 10 ng/ml). The specific activities of these 15 mutants are given in FIG. 4. Raw coagulant activity of these mutants was determined by chromogenic assay. This property is interesting because it would allow smaller or less frequent doses of FVIII to be injected in patients. Moreover, these mutations which confer a higher specific activity might be of major interest in combination with mutations conferring abolition to inhibition by inhibitory antibodies, by allowing to compensate an optional relative loss of activity of said less antigenic mutants.

Example 5: Selection and combination of the best single mutants selected in the secondary screen

Among the 30 single mutants selected in the secondary screen, eight were chosen in order to combine their respective mutations, to obtain a cumulative/additive effect of remarkable properties of each. The selection criteria for these mutants were complex and considered the following parameters:

- at least 25% abolition to inhibition for at least one of the test sera from hemophiliac patients with inhibitors;
- raw coagulant activity at least 100% relative to non-mutated FVIII; and
- reproducibly good level of expression.

The eight selected mutants were mutants **409, 462, 507** and **629** in the A2 domain and mutants **2289, 2294, 2312** and **2316** in the C2 domain. As noted earlier, the selection criterion considered of a high specific activity (coagulant activity relative to expression level), as shown in FIG. 19. This specific activity level had to be constant in the different experiments.

The 28 double mutants resulting from the combination of the eight single mutations **409, 462, 507, 629, 2289, 2294, 2312** and **2316** (six A2 double mutants + six C2 double mutants + sixteen A2-C2 double mutants presented in FIG. 20) were constructed by mutagenesis methods known to one skilled in the art. These mutants were transiently expressed in COS-7 mammalian cells as described in Example 2. Their expression level and their activity level were determined as described in the previous examples, respectively by ELISA and chromogenic assay (mUOD/min). These 28 mutants were then assessed for their abolition to inhibition by antibodies from hemophiliac patients. The A2 double mutants displayed a significant abolition to inhibition for one or all of the antibodies from the patients' sera, whereas the combinations containing C2 domain mutations (six C2 double mutants + sixteen A2-C2 double mutants) displayed an insignificant or null abolition to inhibition.

FIG. 21 shows the specific activities of the six A2 double mutants and their percentage of abolition to inhibition by sera from four hemophiliac patients TD, GC, SL and PR calculated as in Example 4. Especially preferred double mutants significantly abolished antibodies from a minimum of three over the four patients. This illustrates the cumulative effect of the four single mutations in the A2 domain. The choice was therefore based on the combination of the four mutations **409, 507, 462** and **629**. Triple mutants and the quadruple mutant comprising these four mutations **409, 507, 462** and **629** were also constructed.

Residual activity, determined after incubation with inhibitory antibodies, is divided by remained activity after incubation with a non-immune antibody to give the residual activity percentage.

Table 1: Primary screen results; list of 158 Alanine mutants selected for secondary screening, having retained at least 50% of raw activity relative to non-mutated FVIII activity.

Table 2: Secondary screening: Bethesda assays on 30 mutants displaying modified antigenicity towards sera from five hemophiliac patients with inhibitors. Results are expressed as the abolition to inhibition percentage for each mutant as exemplified in FIG. 5.

Table 3: Comparison of specific activity and raw activity relative to non-mutated FVIII activity for the 30 mutants displaying modified antigenicity towards sera from five hemophiliac patients with inhibitors.

Table 4: List of all FVIII double mutants produced from the eight single mutants FVIII409A, FVIII462A, FVIII507A, FVIII629A, FVIII2289A, FVIII2294A, FVIII2312A and FVIII2316A.

Table 5: Chromogenic specific activities and abolition to inhibition percentages towards inhibitory antibodies of six double A2 mutants from sera of four hemophiliac patients TD, GC, SL and PR.

## DESCRIPTION OF THE INVENTION

The present invention provides a solution to resolve a serious complication that occurs in 30% of hemophilia A patients treated with recombinant FVIII: the development of an immune response induced by the treatment and directed against the exogenous recombinant FVIII. The solution provided consists in generating recombinant human FVIII molecules having decreased antigenicity of the epitopes usually recognized by inhibitory antibodies. The FVIII variants of the invention have lost one or more epitopes usually recognized by said antibodies.

The present invention provides other solutions consisting in generating human FVIII variants having an improved specific activity as compared to natural FVIII.

Lastly, the present invention provides with FVIII variants having a greater capacity to be secreted, which is interesting for the production of recombinant FVIII and in a potential gene therapy.

The different properties conferred by the mutations in these variants may be of major interest in combination. In a non-limiting example, mutations which confer a specific activity improvement of a variant could compensate an optional relative loss of activity in variants whose mutations confer an abolition to inhibition by inhibitory antibodies and being therefore less antigenic. In another non-limiting example, mutations which confer a higher capacity to be secreted may be interesting in combination with mutations conferring an abolition to inhibition by inhibitory antibodies, by allowing, for example, to compensate an optional relative loss of secretion of said less antigenic mutants.

In the present document, the following terminology is used to designate a substitution: S409A indicates the substitution of the serine residue at position 409 of SEQ ID No. 3 by an alanine. Substitution refers to the replacement of an amino acid residue by another one selected from the other 19 amino acids or by a non-naturally occurring amino acid. The terms "substitution" and "mutation" are interchangeable. The sign "+" indicates a combination of substitutions.

"Comprise" means that the variant or the fragment thereof has one or more substitutions such as indicated with reference

to SEQ ID No. 3, but that the variant or the fragment thereof may have other modifications, particularly substitutions, deletions or insertions.

“Consists of” means that the variant or the fragment thereof contains only the substitution(s) indicated with reference to SEQ ID No. 3.

“Variant” refers in particular to a polypeptide which differs from a polypeptide represented by sequence SEQ ID No. 3 by at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 or 15 residue(s), preferably by 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 residues.

Amino acids of the A2, A3 or C2 domains of FVIII were systematically substituted by an Alanine. The production of these human FVIII mutants was carried out in mammalian cells. The primary screening of these variants was based on their procoagulant activity. The raw activity of each mutant was measured by chromogenic assay and compared with chromogenic assay of non-mutated human FVIII as reference. The activity of the FVIII variants can be determined by any method known to one skilled in the art, preferably according to method described in example 3 herein after. The FVIII variants selected as being the most active in the primary screen were then assessed for a second feature: loss of antigenicity towards sera from hemophiliac patients selected for their capacity to inhibit FVIII activity. Said secondary screening with said antibodies corresponds to a modified Bethesda assay. The antigenicity modification of the FVIII variants can be determined by any method known to one skilled in the art, preferably according to the method described in example 4 below.

Improved variants could be selected. Not only did some of these candidate medicaments retain a coagulant activity, but they also partially avoided inhibition by inhibitory antibodies from the sera of selected hemophiliac patients. These FVIII have lost one or more epitopes usually recognized by inhibitory antibodies from patients' sera. Furthermore, the candidate medicaments had a specific coagulant activity higher to that of wild-type FVIII. Another interesting feature is that the candidate medicaments displayed an improved secretion capacity.

In one embodiment, the invention therefore relates to recombinant human FVIII variants having lost at least one of the epitopes usually recognized by anti-FVIII antibodies known as “inhibitors”, while retaining a coagulant activity, preferably higher, similar or close to that of non-mutated FVIII.

The present invention describes human FVIII variants comprising at least one substitution of an amino acid by an Alanine or any other amino acid in the C2 and A2 domains.

In particular the invention describes 158 Alanine mutants of human FVIII. “Alanine mutant”, as used herein, denotes a mutant comprising the substitution of an amino acid by an Alanine residue. In particular, said mutants have an Alanine substitution at a residue located among the positions 2316, 2177, 2181, 2182, 2183, 2186, 2189, 2191, 2197, 2199, 2200, 2204, 2205, 2206, 2212, 2213, 2214, 2217, 2221, 2225, 2226, 2235, 2239, 2242, 2244, 2250, 2251, 2252, 2253, 2256, 2258, 2261, 2263, 2264, 2268, 2269, 2270, 2273, 2274, 2275, 2277, 2278, 2280, 2281, 2282, 2284, 2289, 2292, 2294, 2296, 2311, 2312, 2317, 2321 and 2324 of the C2 domain and the positions 378, 383, 391, 398, 399, 400, 403, 406, 407, 408, 409, 410, 413, 414, 415, 416, 417, 421, 429, 432, 440, 442, 444, 445, 449, 452, 454, 455, 462, 464, 468, 481, 486, 490, 491, 493, 494, 496, 497, 498, 499, 500, 507, 512, 517, 518, 519, 520, 523, 524, 526, 530, 532, 534, 539, 540, 543, 550, 552, 556, 559, 562, 567, 568, 573, 578, 588, 592, 596, 597, 600, 601, 602, 604, 607, 611, 621, 624, 628, 629, 632, 633, 640 and 642 of the A2 domain.

The positions of the residues are indicated with reference to the protein sequence of the 2332 amino-acid human FVIII, as illustrated in SEQ ID No. 3.

The invention relates to a human FVIII variant or a biologically active derivative thereof comprising a substitution of at least one amino acid of the C2 domain selected from the group consisting of the residues at positions 2316, 2177, 2181, 2182, 2183, 2186, 2189, 2191, 2197, 2199, 2200, 2204, 2205, 2206, 2212, 2213, 2214, 2217, 2221, 2225, 2226, 2235, 2239, 2242, 2244, 2250, 2251, 2252, 2253, 2256, 2258, 2261, 2263, 2264, 2268, 2269, 2270, 2273, 2274, 2275, 2277, 2278, 2280, 2281, 2282, 2284, 2289, 2292, 2294, 2296, 2311, 2312, 2317, 2321 and 2324. The variant can further comprise a substitution of at least one residue at position 2175, 2195, 2196, 2202, 2215 and 2222. The residue can be substituted by an amino acid selected from an Alanine, a Methionine, a Serine, a Glycine, and a Leucine, preferably an Alanine. Said amino acids, among the twenty naturally occurring amino acids, are known to decrease the antigenicity of a protein. The substitution or substitutions at these positions, in particular by an Alanine, result in an improved FVIII variant, in particular having lost one or more epitopes recognized by inhibitory antibodies and having retained its procoagulant activity. The present invention also relates to a FVIII light chain comprising a substitution of at least one amino acid of the C2 domain selected from the group consisting of the residues at positions 2316, 2177, 2181, 2182, 2183, 2186, 2189, 2191, 2197, 2199, 2200, 2204, 2205, 2206, 2212, 2213, 2214, 2217, 2221, 2225, 2226, 2235, 2239, 2242, 2244, 2250, 2251, 2252, 2253, 2256, 2258, 2261, 2263, 2264, 2268, 2269, 2270, 2273, 2274, 2275, 2277, 2278, 2280, 2281, 2282, 2284, 2289, 2292, 2294, 2296, 2311, 2312, 2317, 2321 and 2324. This light chain can further comprise a substitution of at least one residue at position 2175, 2195, 2196, 2202, 2215 and 2222.

The invention further relates to a human FVIII variant or a biologically active derivative thereof comprising or containing a substitution of at least one amino acid of the A2 domain, preferably selected from the group consisting of the residues at positions 378, 383, 391, 398, 399, 400, 403, 406, 407, 408, 409, 410, 413, 414, 415, 416, 417, 421, 429, 432, 440, 442, 444, 445, 449, 452, 454, 455, 462, 464, 468, 481, 486, 490, 491, 493, 494, 496, 497, 498, 499, 500, 507, 512, 517, 518, 519, 520, 523, 524, 526, 530, 532, 534, 539, 540, 543, 550, 552, 556, 559, 562, 567, 568, 573, 578, 588, 592, 596, 597, 600, 601, 602, 604, 607, 611, 621, 624, 628, 629, 632, 633, 640 and 642. The variant can further comprise a substitution of at least one residue at position 377, 379, 405, 434, 437, 485, 488, 489, 492, 495, 501, 508 and 623. The residue can be substituted by an amino acid selected from an Alanine, a Methionine, a Serine, a Glycine, and a Leucine, preferably an Alanine. The substitution or substitutions at these positions, in particular by an Alanine, result in an improved FVIII variant, in particular having lost one or more epitopes recognized by inhibitory antibodies and having retained its procoagulant activity. The present invention also relates to a FVIII heavy chain, optionally which totally or partially lacks the B domain, comprising a substitution of at least one amino acid of the A2 domain selected from the group consisting of the residues at positions 378, 383, 391, 398, 399, 400, 403, 406, 407, 408, 409, 410, 413, 414, 415, 416, 417, 421, 429, 432, 440, 442, 444, 445, 449, 452, 454, 455, 462, 464, 468, 481, 486, 490, 491, 493, 494, 496, 497, 498, 499, 500, 507, 512, 517, 518, 519, 520, 523, 524, 526, 530, 532, 534, 539, 540, 543, 550, 552, 556, 559, 562, 567, 568, 573, 578, 588, 592, 596, 597, 600, 601, 602, 604, 607, 611, 621, 624, 628, 629, 632, 633, 640 and 642. The variant can further comprise a

substitution of at least one residue at position 377, 379, 405, 434, 437, 485, 488, 489, 492, 495, 501, 508 and 623.

The invention further relates to a human FVIII variant or a biologically active derivative thereof comprising a substitution of at least one amino acid comprising or containing a substitution of at least one amino acid selected from the group consisting of the residues at positions 2316, 2177, 2181, 2182, 2183, 2186, 2189, 2191, 2197, 2199, 2200, 2204, 2205, 2206, 2212, 2213, 2214, 2217, 2221, 2225, 2226, 2235, 2239, 2242, 2244, 2250, 2251, 2252, 2253, 2256, 2258, 2261, 2263, 2264, 2268, 2269, 2270, 2273, 2274, 2275, 2277, 2278, 2280, 2281, 2282, 2284, 2289, 2292, 2294, 2296, 2311, 2312, 2317, 2321 and 2324 of the C2 domain and the residues at positions 378, 383, 391, 398, 399, 400, 403, 406, 407, 408, 409, 410, 413, 414, 415, 416, 417, 421, 429, 432, 440, 442, 444, 445, 449, 452, 454, 455, 462, 464, 468, 481, 486, 490, 491, 493, 494, 496, 497, 498, 499, 500, 507, 512, 517, 518, 519, 520, 523, 524, 526, 530, 532, 534, 539, 540, 543, 550, 552, 556, 559, 562, 567, 568, 573, 578, 588, 592, 596, 597, 600, 601, 602, 604, 607, 611, 621, 624, 628, 629, 632, 633, 640 and 642 of the A2 domain. In a particular embodiment, the human FVIII variant or the biologically active derivative thereof further comprises a substitution of at least one amino acid selected from the group consisting of the residues at positions 2175, 2195, 2196, 2202, 2215 and 2222 of the C2 domain and the residues at positions 377, 379, 405, 434, 437, 485, 488, 489, 492, 495, 501, 508 and 623 of the A2 domain. In a particular embodiment, the human FVIII variant or the biologically active derivative thereof comprises the substitution of at least two, three, four, five, six, seven, eight, nine, ten, eleven, twelve, thirteen, fourteen or fifteen amino acids, preferably selected from the aforementioned groups.

In a preferred embodiment, the invention relates to an improved human FVIII variant or a biologically active derivative thereof having a decreased antigenicity and comprising the substitution of at least one amino acid selected from the group consisting of the residues at positions 2206, 2212, 2226, 2244, 2261, 2275, 2280, 2281, 2282, 2289, 2294, 2311, 2312, and 2316 of the C2 domain and the residues at positions 400, 403, 409, 414, 421, 462, 486, 493, 494, 496, 507, 518, 562, and 629 of the A2 domain. In another embodiment, said variant can further comprise a substitution of at least one amino acid selected from the group consisting of the residue at position 2202 of the C2 domain and the residue at position 437 of the A2 domain. The residue can be substituted by an amino acid selected from an Alanine, a Methionine, a Serine, a Glycine, and a Leucine, preferably an Alanine. In a particular embodiment, said human FVIII variant or biologically active derivative thereof has a single substitution. Said single substitution is preferably selected from the group consisting of the substitutions L400A, L400M, L400S, L400G, D403A, D403M, D403S, D403G, D403L, S409A, S409M, S409G, S409L, N414A, N414M, N414S, N414G, N414L, R421A, R421M, R421S, R421G, R421L, L462A, L462M, L462S, L462G, L486A, L486M, L486G, K493M, K493S, K493G, K493L, G494A, G494M, G494L, K496A, K496S, K496G, K496L, E507A, E507M, E507S, E507L, E518A, E518M, E518S, E518G, E518L, R562A, R562M, R562S, R562G, R562L, V629A, V629M, V629S, V629G and V629L in the A2 domain and the substitutions S2206A, S2206G, S2206M, S2206L, L2212A, L2212M, L2212S, L2212G, P2226A, P2226M, P2226S, P2226G, P2226L, T2244A, T2244M, T2244S, T2244G, T2244L, L2261A, L2261M, L2261S, L2261G, F2275A, F2275M, F2275S, F2275G, F2275L, V2280A, V2280M, V2280S, V2280G, V2280L, K2281A, K2281M, K2281S, K2281G, K2281L, V2282A, V2282M, V2282S, V2282G, V2282L, S2289A, S2289M, S2289G,

S2289L, V2294A, V2294M, V2294S, V2294G, V2294L, Q2311A, Q2311M, Q2311S, Q2311G, Q2311L, S2312A, S2312M, S2312G, S2312L, Q2316A, Q2316M, Q2316S, Q2316G and Q2316L in the C2 domain. In another embodiment, the invention relates to a human FVIII variant or a biologically active derivative thereof comprising at least one substitution selected from the group consisting of the substitutions L400A, L400M, L400S, L400G, D403A, D403M, D403S, D403G, D403L, S409A, S409M, S409G, S409L, N414A, N414M, N414S, N414G, N414L, R421A, R421M, R421S, R421G, R421L, L462A, L462M, L462S, L462G, L486A, L486M, L486G, K493M, K493S, K493G, K493L, G494A, G494M, G494L, K496A, K496S, K496G, K496L, E507A, E507M, E507S, E507L, E518A, E518M, E518S, E518G, E518L, R562A, R562M, R562S, R562G, R562L, V629A, V629M, V629S, V629G and V629L in the A2 domain and the substitutions S2206A, S2206G, S2206M, S2206L, L2212A, L2212M, L2212S, L2212G, P2226A, P2226M, P2226S, P2226G, P2226L, T2244A, T2244M, T2244S, T2244G, T2244L, L2261A, L2261M, L2261S, L2261G, F2275A, F2275M, F2275S, F2275G, F2275L, V2280A, V2280M, V2280S, V2280G, V2280L, K2281A, K2281M, K2281S, K2281G, K2281L, V2282A, V2282M, V2282S, V2282G, V2282L, S2289A, S2289M, S2289G, S2289L, V2294A, V2294M, V2294S, V2294G, V2294L, Q2311A, Q2311M, Q2311S, Q2311G, Q2311L, S2312A, S2312M, S2312G, S2312L, Q2316A, Q2316M, Q2316S, Q2316G and Q2316L in the C2 domain. Said FVIII variants have lost one or more epitopes usually recognized by said antibodies and therefore have decreased antigenicity as compared to non-mutated human FVIII. Furthermore, they have retained at least 50%, preferably at least 60 or 75%, of raw activity relative to non-mutated human FVIII.

In a still more preferred embodiment, the invention relates to an improved human FVIII variant or a biologically active derivative thereof having a decreased antigenicity and having retained at least 100% of raw activity as compared to non-mutated human FVIII, and comprising the substitution of at least one amino acid selected from the group consisting of the residues at positions 409, 462, 507, and 629 of the A2 domain and the residues at positions 2289, 2294, 2312, and 2316 of the C2 domain. In another embodiment, said variant can further comprise a substitution of at least one amino acid selected from the group consisting of the residue at position 2202 of the C2 domain and the residue at position 437 of the A2 domain. The residue can be substituted by an amino acid selected from an Alanine, a Methionine, a Serine, a Glycine, and a Leucine, preferably an Alanine. In a particular embodiment, said human FVIII variant or biologically active derivative thereof has a single substitution. Said substitution is preferably selected from the group consisting of the substitutions S409A, S409M, S409G, S409L, L462A, L462M, L462S, L462G, E507A, E507M, E507S, E507L, V629A, V629M, V629S, V629G, V629L, S2289A, S2289M, S2289G, S2289L, V2294A, V2294M, V2294S, V2294G, V2294L, S2312A, S2312M, S2312G, S2312L, Q2316A, Q2316M, Q2316S, Q2316G and Q2316L. In another embodiment, the invention relates to a human FVIII variant or a biologically active derivative thereof comprising at least one substitution selected from the group consisting of the substitutions S409A, S409M, S409G, S409L, L462A, L462M, L462S, L462G, E507A, E507M, E507S, E507L, V629A, V629M, V629S, V629G, V629L, S2289A, S2289M, S2289G, S2289L, V2294A, V2294M, V2294S, V2294G, V2294L, S2312A, S2312M, S2312G, S2312L, Q2316A, Q2316M, Q2316S, Q2316G and Q2316L.

In a further embodiment, the invention relates to an improved human FVIII variant or a biologically active derivative thereof having a decreased antigenicity and comprising the combination of two substitutions selected from the group consisting of 409+462, 409+507, 462+507, 409+629, 462+ 5 629 and 507+629, preferably 409+462, 409+507 and 462+507. In a particular embodiment, said human FVIII variant or biologically active derivative thereof comprises the combination of two substitutions selected from the group consisting of S409A+L462A, S409A+L462M, S409A+L462S, S409A+ 10 L462G, S409A+E507A, S409A+E507M, S409A+E507S, S409A+E507G, S409A+E507L, S409A+V629A, S409A+V629M, S409A+V629S, S409A+V629G, S409A+V629L, S409M+L462A, S409M+L462M, S409M+L462S, S409M+L462G, S409M+E507A, S409M+E507M, S409M+E507S, 15 S409M+E507G, S409M+E507L, S409M+V629A, S409M+V629M, S409M+V629S, S409M+V629G, S409M+V629L, S409G+L462A, S409G+L462M, S409G+L462S, S409G+L462G, S409G+E507A, S409G+E507M, S409G+E507S, S409G+E507G, S409G+E507L, S409G+V629A, S409G+ 20 V629M, S409G+V629S, S409G+V629G, S409G+V629L, S409L+L462A, S409L+L462M, S409L+L462S, S409L+L462G, S409L+E507A, S409L+E507M, S409L+E507S, S409L+E507G, S409L+E507L, S409L+V629A, S409L+V629M, S409L+V629S, S409L+V629G, S409L+V629L, 25 L462A+E507A, L462A+E507M, L462A+E507S, L462A+E507G, L462A+E507L, L462A+V629A, L462A+V629M, L462A+V629S, L462A+V629G, L462A+V629L, L462M+E507A, L462M+E507M, L462M+E507S, L462M+E507G, L462M+E507L, L462M+V629A, L462M+V629M, 30 L462M+V629S, L462M+V629G, L462M+V629L, L462S+E507A, L462S+E507M, L462S+E507S, L462S+E507G, L462S+E507L, L462S+V629A, L462S+V629M, L462S+V629S, L462S+V629G, L462S+V629L, L462G+E507A, L462G+E507M, L462G+E507S, L462G+E507G, L462G+ 35 E507L, L462G+V629A, L462G+V629M, L462G+V629S, L462G+V629G, L462G+V629L, E507A+V629A, E507A+V629M, E507A+V629S, E507A+V629G, E507A+V629L, E507M+V629A, E507M+V629M, E507M+V629S, E507M+V629G, E507M+V629L, E507S+V629A, E507S+ 40 V629M, E507S+V629S, E507S+V629G, E507S+V629L, E507G+V629A, E507G+V629M, E507G+V629S, E507G+V629G, E507G+V629L, E507L+V629A, E507L+V629M, E507L+V629S, E507L+V629G and E507L+V629L, preferably in the group consisting of S409A+L462A, S409A+ 45 L462M, S409A+L462S, S409A+L462G, S409A+E507A, S409A+E507M, S409A+E507S, S409A+E507G, S409A+E507L, S409M+L462A, S409M+L462M, S409M+L462S, S409M+L462G, S409M+E507A, S409M+E507M, S409M+E507S, S409M+E507G, S409M+E507L, S409G+L462A, 50 S409G+L462M, S409G+L462S, S409G+L462G, S409G+E507A, S409G+E507M, S409G+E507S, S409G+E507G, S409G+E507L, S409L+L462A, S409L+L462M, S409L+L462S, S409L+L462G, S409L+E507A, S409L+E507M, S409L+E507S, S409L+E507G, S409L+E507L, L462A+ 55 E507A, L462A+E507M, L462A+E507S, L462A+E507G, L462A+E507L, L462M+E507A, L462M+E507M, L462M+E507S, L462M+E507G, L462M+E507L, L462S+E507A, L462S+E507M, L462S+E507S, L462S+E507G, L462S+E507L, L462G+E507A, L462G+E507M, L462G+E507S, 60 L462G+E507G and L462G+E507L.

In yet another embodiment, the invention relates to an improved human FVIII variant or a biologically active derivative thereof comprising the combination of three substitutions selected from the group consisting of 409+462+507, 462+ 65 507+629, 409+462+629, 409+507+629, preferably 409+462+507. In a particular embodiment, said human FVIII

variant or biologically active derivative thereof comprises the combination of three substitutions selected from the group consisting of S409A+L462A+E507A, S409A+L462A+E507M, S409A+L462A+E507S, S409A+L462A+E507G, 5 S409A+L462A+E507L, S409A+L462M+E507A, S409A+L462M+E507M, S409A+L462M+E507S, S409A+L462M+E507G, S409A+L462M+E507L, S409A+L462S+E507A, S409A+L462S+E507M, S409A+L462S+E507S, S409A+L462S+E507G, S409A+L462S+E507L, S409A+L462G+E507A, S409A+L462G+E507M, S409A+L462G+E507S, 10 S409A+L462G+E507G, S409A+L462G+E507L, S409M+L462A+E507A, S409M+L462A+E507M, S409M+L462A+E507S, S409M+L462A+E507G, S409M+L462A+E507L, S409M+L462M+E507A, S409M+L462M+E507M, 15 S409M+L462M+E507S, S409M+L462M+E507G, S409M+L462M+E507L, S409M+L462S+E507A, S409M+L462S+E507M, S409M+L462S+E507S, S409M+L462S+E507G, S409M+L462S+E507L, S409M+L462G+E507A, S409M+L462G+E507M, S409M+L462G+E507S, S409M+L462G+E507G, 20 S409M+L462G+E507L, S409G+L462A+E507A, S409G+L462A+E507M, S409G+L462A+E507S, S409G+L462A+E507G, S409G+L462A+E507L, S409G+L462M+E507A, S409G+L462M+E507M, S409G+L462M+E507S, S409G+L462M+E507G, S409G+L462M+E507L, S409G+L462S+E507A, S409G+L462S+E507M, S409G+L462S+E507S, S409G+L462S+E507G, S409G+L462S+E507L, 25 S409G+L462G+E507A, S409G+L462G+E507M, S409G+L462G+E507S, S409G+L462G+E507G, S409G+L462G+E507L, S409L+L462A+E507A, S409L+L462A+E507M, S409L+L462A+E507S, S409L+L462A+E507G, S409L+L462A+E507L, S409L+L462M+E507A, S409L+L462M+E507M, S409L+L462M+E507S, S409L+L462M+E507G, S409L+L462M+E507L, S409L+L462S+E507A, S409L+L462S+E507M, S409L+L462S+E507S, S409L+L462S+E507G, S409L+L462S+E507L, 30 S409L+L462G+E507A, S409L+L462G+E507M, S409L+L462G+E507S, S409L+L462G+E507G, S409L+L462G+E507L, S409A+L462A+V629A, S409A+L462A+V629M, S409A+L462A+V629S, S409A+L462A+V629G, S409A+L462A+V629L, S409A+L462M+V629A, S409A+L462M+V629M, S409A+L462M+V629S, 35 S409A+L462M+V629G, S409A+L462M+V629L, S409A+L462S+V629A, S409A+L462S+V629M, S409A+L462S+V629S, S409A+L462S+V629G, S409A+L462S+V629L, S409A+L462G+V629A, S409A+L462G+V629M, S409A+L462G+V629S, S409A+L462G+V629G, S409A+L462G+V629L, S409M+L462A+V629A, S409M+L462A+V629M, S409M+L462A+V629S, S409M+L462A+V629G, S409M+L462A+V629L, S409M+L462M+V629A, S409M+L462M+V629M, S409M+L462M+V629S, 40 S409M+L462M+V629G, S409M+L462M+V629L, S409M+L462S+V629A, S409M+L462S+V629M, S409M+L462S+V629S, S409M+L462S+V629G, S409M+L462S+V629L, S409M+L462G+V629A, S409M+L462G+V629M, S409M+L462G+V629S, S409M+L462G+V629G, S409M+L462G+V629L, S409G+L462A+V629A, S409G+L462A+V629M, S409G+L462A+V629S, S409G+L462A+V629G, S409G+L462A+V629L, S409G+L462M+V629A, S409G+L462M+V629M, S409G+L462M+V629S, S409G+L462M+V629G, S409G+L462M+V629L, S409G+L462S+V629A, S409G+L462S+V629M, S409G+L462S+V629S, S409G+L462S+V629G, S409G+L462S+V629L, S409G+L462G+V629A, S409G+L462G+V629M, S409G+L462G+V629S, S409G+L462G+V629G, S409G+L462G+V629L, S409L+L462A+V629A, S409L+L462A+V629M, S409L+L462A+V629L, S409L+L462A+V629S, S409L+L462A+V629G, S409L+L462A+V629M, S409L+L462A+V629L, S409L+L462M+V629A, S409L+L462M+V629M, S409L+L462M+V629S, S409L+L462M+V629G, S409L+L462M+

V629L, S409L+L462S+V629A, S409L+L462S+V629M, S409L+L462S+V629S, S409L+L462S+V629G, S409L+L462S+V629L, S409L+L462G+V629A, S409L+L462G+V629M, S409L+L462G+V629S, S409L+L462G+V629G, S409L+L462G+V629L, S409A+E507A+V629A, S409A+E507A+V629M, S409A+E507A+V629S, S409A+E507A+V629G, S409A+E507A+V629L, S409A+E507M+V629A, S409A+E507M+V629M, S409A+E507M+V629S, S409A+E507M+V629G, S409A+E507M+V629L, S409A+E507S+V629A, S409A+E507S+V629M, S409A+E507S+V629S, S409A+E507S+V629G, S409A+E507S+V629L, S409A+E507G+V629A, S409A+E507G+V629M, S409A+E507G+V629S, S409A+E507G+V629G, S409A+E507G+V629L, S409A+E507L+V629A, S409A+E507L+V629M, S409A+E507L+V629S, S409A+E507L+V629G, S409A+E507L+V629L, S409M+E507A+V629A, S409M+E507A+V629M, S409M+E507A+V629S, S409M+E507A+V629G, S409M+E507A+V629L, S409M+E507M+V629A, S409M+E507M+V629M, S409M+E507M+V629S, S409M+E507M+V629G, S409M+E507M+V629L, S409M+E507S+V629A, S409M+E507S+V629M, S409M+E507S+V629S, S409M+E507S+V629G, S409M+E507S+V629L, S409M+E507G+V629A, S409M+E507G+V629M, S409M+E507G+V629S, S409M+E507G+V629G, S409M+E507G+V629L, S409M+E507L+V629A, S409M+E507L+V629M, S409M+E507L+V629S, S409M+E507L+V629G, S409M+E507L+V629L, S409G+E507A+V629A, S409G+E507A+V629M, S409G+E507A+V629S, S409G+E507A+V629G, S409G+E507A+V629L, S409G+E507M+V629A, S409G+E507M+V629M, S409G+E507M+V629S, S409G+E507M+V629G, S409G+E507M+V629L, S409G+E507S+V629A, S409G+E507S+V629M, S409G+E507S+V629S, S409G+E507S+V629G, S409G+E507S+V629L, S409G+E507G+V629A, S409G+E507G+V629M, S409G+E507G+V629S, S409G+E507G+V629L, S409G+E507L+V629A, S409G+E507L+V629M, S409G+E507L+V629S, S409G+E507L+V629G, S409L+E507A+V629A, S409L+E507A+V629M, S409L+E507A+V629S, S409L+E507A+V629G, S409L+E507M+V629A, S409L+E507M+V629M, S409L+E507M+V629S, S409L+E507M+V629G, S409L+E507M+V629L, S409L+E507S+V629A, S409L+E507S+V629M, S409L+E507S+V629S, S409L+E507S+V629G, S409L+E507S+V629L, S409L+E507G+V629A, S409L+E507G+V629M, S409L+E507G+V629S, S409L+E507G+V629G, S409L+E507G+V629L, S409L+E507L+V629A, S409L+E507L+V629M, S409L+E507L+V629S, S409L+E507L+V629G, S409L+E507L+V629L, L462A+E507A+V629A, L462A+E507A+V629M, L462A+E507A+V629S, L462A+E507A+V629G, L462A+E507A+V629L, L462A+E507M+V629A, L462A+E507M+V629M, L462A+E507M+V629S, L462A+E507M+V629G, L462A+E507M+V629L, L462A+E507S+V629A, L462A+E507S+V629M, L462A+E507S+V629S, L462A+E507S+V629G, L462A+E507S+V629L, L462A+E507G+V629A, L462A+E507G+V629M, L462A+E507G+V629S, L462A+E507G+V629G, L462A+E507G+V629L, L462A+E507L+V629A, L462A+E507L+V629M, L462A+E507L+V629S, L462A+E507L+V629G, L462A+E507L+V629L, L462M+E507A+V629A, L462M+E507A+V629M, L462M+E507A+V629S, L462M+E507A+V629G, L462M+E507A+V629L, L462M+E507M+V629A, L462M+E507M+V629M, L462M+E507M+V629S, L462M+E507M+V629G, L462M+E507M+V629L, L462M+E507S+V629A, L462M+E507S+V629M, L462M+E507S+V629S, L462M+E507S+V629G, L462M+E507S+V629L, L462M+E507G+V629A, L462M+E507G+V629M, L462M+E507G+V629S, L462M+E507G+V629G, L462M+

E507G+V629L, L462M+E507L+V629A, L462M+E507L+V629M, L462M+E507L+V629S, L462M+E507L+V629G, L462M+E507L+V629L, L462S+E507A+V629A, L462S+E507A+V629M, L462S+E507A+V629S, L462S+E507A+V629G, L462S+E507A+V629L, L462S+E507M+V629A, L462S+E507M+V629M, L462S+E507M+V629S, L462S+E507M+V629G, L462S+E507M+V629L, L462S+E507S+V629A, L462S+E507S+V629M, L462S+E507S+V629S, L462S+E507S+V629G, L462S+E507S+V629L, L462S+E507G+V629A, L462S+E507G+V629M, L462S+E507G+V629S, L462S+E507G+V629L, L462S+E507L+V629A, L462S+E507L+V629M, L462S+E507L+V629S, L462S+E507L+V629G, L462S+E507L+V629L, L462G+E507A+V629A, L462G+E507A+V629M, L462G+E507A+V629S, L462G+E507A+V629G, L462G+E507M+V629A, L462G+E507M+V629M, L462G+E507M+V629S, L462G+E507M+V629G, L462G+E507M+V629L, L462G+E507S+V629A, L462G+E507S+V629M, L462G+E507S+V629S, L462G+E507S+V629G, L462G+E507S+V629L, L462G+E507G+V629A, L462G+E507G+V629M, L462G+E507G+V629S, L462G+E507G+V629L, L462G+E507L+V629A, L462G+E507L+V629M, L462G+E507L+V629S, L462G+E507L+V629G and L462G+E507L+V629L, preferably in the group consisting of S409A+L462A+E507A, S409A+L462A+E507M, S409A+L462A+E507S, S409A+L462A+E507G, S409A+L462A+E507L, S409A+L462M+E507A, S409A+L462M+E507M, S409A+L462M+E507S, S409A+L462M+E507G, S409A+L462M+E507L, S409A+L462S+E507A, S409A+L462S+E507M, S409A+L462S+E507S, S409A+L462S+E507G, S409A+L462S+E507L, S409A+L462G+E507A, S409A+L462G+E507M, S409A+L462G+E507S, S409A+L462G+E507G, S409A+L462G+E507L, S409M+L462A+E507A, S409M+L462A+E507M, S409M+L462A+E507S, S409M+L462A+E507G, S409M+L462A+E507L, S409M+L462M+E507A, S409M+L462M+E507M, S409M+L462M+E507S, S409M+L462M+E507G, S409M+L462M+E507L, S409M+L462S+E507A, S409M+L462S+E507M, S409M+L462S+E507S, S409M+L462S+E507G, S409M+L462S+E507L, S409M+L462G+E507A, S409M+L462G+E507M, S409M+L462G+E507S, S409M+L462G+E507G, S409M+L462G+E507L, S409G+L462A+E507A, S409G+L462A+E507M, S409G+L462A+E507S, S409G+L462A+E507G, S409G+L462A+E507L, S409G+L462M+E507A, S409G+L462M+E507M, S409G+L462M+E507S, S409G+L462M+E507G, S409G+L462M+E507L, S409G+L462S+E507A, S409G+L462S+E507M, S409G+L462S+E507S, S409G+L462S+E507G, S409G+L462S+E507L, S409G+L462G+E507A, S409G+L462G+E507M, S409G+L462G+E507S, S409G+L462G+E507G, S409G+L462G+E507L, S409L+L462A+E507A, S409L+L462A+E507M, S409L+L462A+E507S, S409L+L462A+E507G, S409L+L462A+E507L, S409L+L462M+E507A, S409L+L462M+E507M, S409L+L462M+E507S, S409L+L462M+E507G, S409L+L462M+E507L, S409L+L462S+E507A, S409L+L462S+E507M, S409L+L462S+E507S, S409L+L462S+E507G, S409L+L462S+E507L, S409L+L462G+E507A, S409L+L462G+E507M, S409L+L462G+E507S, S409L+L462G+E507G and S409L+L462G+E507L.

In another particular embodiment, the invention relates to an improved human FVIII variant or a biologically active derivative thereof comprising the combination of four substitutions at positions 409, 462, 507 and 629. In a particular embodiment, said human FVIII variant or biologically active derivative thereof comprises the combination of four substitutions selected from the group consisting of S409A+L462A+E507A+V629A, S409A+L462A+E507A+V629M,





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L462M+E507M+V629G, S409G+L462M+E507M+V629L, S409G+L462M+E507S+V629A, S409G+L462M+E507S+V629M, S409G+L462M+E507S+V629S, S409G+L462M+E507S+V629G, S409G+L462M+E507S+V629L, S409G+L462M+E507G+V629A, S409G+L462M+E507G+V629M, S409G+L462M+E507G+V629S, S409G+L462M+E507G+V629G, S409G+L462M+E507G+V629L, S409G+L462M+E507L+V629A, S409G+L462M+E507L+V629M, S409G+L462M+E507L+V629S, S409G+L462M+E507L+V629G, S409G+L462M+E507L+V629L, S409G+L462S+E507A+V629A, S409G+L462S+E507A+V629M, S409G+L462S+E507A+V629S, S409G+L462S+E507A+V629G, S409G+L462S+E507A+V629L, S409G+L462S+E507M+V629A, S409G+L462S+E507M+V629M, S409G+L462S+E507M+V629S, S409G+L462S+E507M+V629G, S409G+L462S+E507M+V629L, S409G+L462S+E507S+V629A, S409G+L462S+E507S+V629M, S409G+L462S+E507S+V629S, S409G+L462S+E507S+V629G, S409G+L462S+E507S+V629L, S409G+L462S+E507L+V629A, S409G+L462S+E507L+V629M, S409G+L462S+E507L+V629S, S409G+L462S+E507L+V629G, S409G+L462S+E507L+V629L, S409G+L462G+E507A+V629A, S409G+L462G+E507A+V629M, S409G+L462G+E507A+V629S, S409G+L462G+E507A+V629G, S409G+L462G+E507A+V629L, S409G+L462G+E507M+V629A, S409G+L462G+E507M+V629M, S409G+L462G+E507M+V629S, S409G+L462G+E507M+V629G, S409G+L462G+E507M+V629L, S409G+L462G+E507S+V629A, S409G+L462G+E507S+V629M, S409G+L462G+E507S+V629S, S409G+L462G+E507S+V629G, S409G+L462G+E507S+V629L, S409G+L462G+E507G+V629A, S409G+L462G+E507G+V629M, S409G+L462G+E507G+V629S, S409G+L462G+E507G+V629G, S409G+L462G+E507G+V629L, S409G+L462G+E507L+V629A, S409G+L462G+E507L+V629M, S409G+L462G+E507L+V629S, S409G+L462G+E507L+V629G, S409G+L462G+E507L+V629L, S409L+L462A+E507A+V629A, S409L+L462A+E507A+V629M, S409L+L462A+E507A+V629S, S409L+L462A+E507A+V629G, S409L+L462A+E507A+V629L, S409L+L462A+E507M+V629A, S409L+L462A+E507M+V629M, S409L+L462A+E507M+V629S, S409L+L462A+E507M+V629G, S409L+L462A+E507M+V629L, S409L+L462A+E507S+V629A, S409L+L462A+E507S+V629M, S409L+L462A+E507S+V629S, S409L+L462A+E507S+V629G, S409L+L462A+E507S+V629L, S409L+L462A+E507G+V629A, S409L+L462A+E507G+V629M, S409L+L462A+E507G+V629S, S409L+L462A+E507G+V629G, S409L+L462A+E507L+V629A, S409L+L462A+E507L+V629M, S409L+L462A+E507L+V629S, S409L+L462A+E507L+V629G, S409L+L462A+E507L+V629L, S409L+L462M+E507A+V629A, S409L+L462M+E507A+V629M, S409L+L462M+E507A+V629S, S409L+L462M+E507A+V629G, S409L+L462M+E507A+V629L, S409L+L462M+E507M+V629A, S409L+L462M+E507M+V629M, S409L+L462M+E507M+V629S, S409L+L462M+E507M+V629G, S409L+L462M+E507M+V629L, S409L+L462M+E507S+V629A, S409L+L462M+E507S+V629M, S409L+L462M+E507S+V629S, S409L+L462M+E507S+V629G, S409L+L462M+E507S+V629L, S409L+L462M+E507G+V629A, S409L+L462M+E507G+V629M, S409L+L462M+E507G+V629S, S409L+L462M+E507G+V629G, S409L+L462M+E507G+V629L, S409L+L462M+E507L+V629A, S409L+L462M+E507L+V629M, S409L+L462M+E507L+V629S, S409L+L462M+E507L+V629G, S409L+L462M+E507L+V629L, S409L+L462S+

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E507A+V629A, S409L+L462S+E507A+V629M, S409L+L462S+E507A+V629S, S409L+L462S+E507A+V629G, S409L+L462S+E507A+V629L, S409L+L462S+E507M+V629A, S409L+L462S+E507M+V629M, S409L+L462S+E507M+V629S, S409L+L462S+E507M+V629G, S409L+L462S+E507M+V629L, S409L+L462S+E507S+V629A, S409L+L462S+E507S+V629M, S409L+L462S+E507S+V629S, S409L+L462S+E507S+V629G, S409L+L462S+E507S+V629L, S409L+L462S+E507G+V629A, S409L+L462S+E507G+V629M, S409L+L462S+E507G+V629S, S409L+L462S+E507G+V629G, S409L+L462S+E507G+V629L, S409L+L462S+E507L+V629A, S409L+L462S+E507L+V629M, S409L+L462S+E507L+V629S, S409L+L462S+E507L+V629G, S409L+L462S+E507L+V629L, S409L+L462G+E507A+V629A, S409L+L462G+E507A+V629M, S409L+L462G+E507A+V629S, S409L+L462G+E507A+V629G, S409L+L462G+E507A+V629L, S409L+L462G+E507M+V629A, S409L+L462G+E507M+V629M, S409L+L462G+E507M+V629S, S409L+L462G+E507M+V629G, S409L+L462G+E507M+V629L, S409L+L462G+E507S+V629A, S409L+L462G+E507S+V629M, S409L+L462G+E507S+V629S, S409L+L462G+E507S+V629G, S409L+L462G+E507S+V629L, S409L+L462G+E507G+V629A, S409L+L462G+E507G+V629M, S409L+L462G+E507G+V629S, S409L+L462G+E507G+V629G, S409L+L462G+E507G+V629L, S409L+L462G+E507L+V629A, S409L+L462G+E507L+V629M, S409L+L462G+E507L+V629S, S409L+L462G+E507L+V629G, S409L+L462G+E507L+V629L.

In a further preferred embodiment, the invention relates to an improved human FVIII variant or a biologically active derivative thereof having an improved specific activity and comprising the substitution of at least one amino acid selected from the group consisting of the residues at positions 2177, 2183, 2186, 2191, 2204, 2205, 2206, 2213, 2217, 2235, 2258, 2264, 2268 and 2269 of the C2 domain. Said variant can further comprise the substitution of the amino acid at position 2196 of the C2 domain. Moreover, said mutations which confer a higher specific activity may prove to be of great interest in combination with mutations conferring abolition to inhibition by inhibitory antibodies, by allowing, for example, to compensate an optional relative loss of activity of said less antigenic mutants. Thus, in a particular embodiment, the invention relates to an improved human FVIII variant or a biologically active derivative thereof comprising the substitution of at least one amino acid selected from the group consisting of the residues at positions 400, 403, 409, 414, 421, 462, 486, 493, 494, 496, 507, 518, 562, and 629 of the A2 domain and the residues at positions 2206, 2212, 2226, 2244, 2261, 2275, 2280, 2281, 2282, 2289, 2294, 2311, 2312, and 2316 of the C2 domain, and further comprising a substitution of at least one amino acid selected from the group consisting of the residues at positions 2177, 2183, 2186, 2191, 2204, 2205, 2213, 2217, 2235, 2258, 2264, 2268 and 2269 of the C2 domain. Preferably, said variant comprises the substitution of at least one amino acid selected from the group consisting of the residues at positions 409, 462, 507 and 629 of the A2 domain and the residues at positions 2289, 2294, 2312 and 2316 of the C2 domain, and further comprises a substitution of at least one amino acid selected from the group consisting of the residues at positions 2177, 2183, 2186, 2191, 2204, 2205, 2213, 2217, 2235, 2258, 2264, 2268 and 2269 of the C2 domain.

In an additional preferred embodiment, the invention relates to an improved human FVIII variant or a biologically active derivative thereof having an improved capacity to be secreted and comprising the substitution of at least one amino

acid selected from the group consisting of the residues at positions 2199, 2200, 2215, 2251, 2252, 2278, and 2316 of the C2 domain. Said variant can further comprise the substitution of the amino acid at position 2175 of the C2 domain. Furthermore, said mutations which confer higher capacity to be secreted may prove to be of great interest in combination with mutations conferring abolition to inhibition by inhibitory antibodies, by allowing, for example, to compensate an optional relative loss of secretion of said less antigenic mutants. Thus, in a particular embodiment, the invention relates to an improved human FVIII variant or a biologically active derivative thereof comprising the substitution of at least one amino acid selected from the group consisting of the residues at positions 400, 403, 409, 414, 421, 462, 486, 493, 494, 496, 507, 518, 562, and 629 of the A2 domain and the residues at positions 2206, 2212, 2226, 2244, 2261, 2275, 2280, 2281, 2282, 2289, 2294, 2311, 2312, and 2316 of the C2 domain, and further comprising a substitution of at least one amino acid selected from the group consisting of the residues at positions 2175, 2199, 2200, 2215, 2251, 2252 and 2278 of the C2 domain. In a preferred manner, said variant comprises the substitution of at least one amino acid selected from the group consisting of the residues at positions 409, 462, 507 and 629 of the A2 domain and the residues at positions 2289, 2294, 2312 and 2316 of the C2 domain, and further comprises a substitution of at least one amino acid selected from the group consisting of the residues at positions 2175, 2199, 2200, 2215, 2251, 2252 and 2278 of the C2 domain.

The broad production of mutants having retained at least 50% of FVIII activity also makes it possible to encompass their use in the context of analyzing additional functions of the protein. In addition to a modulation of its immunogenicity, secretion and specific activity, the following FVIII properties might be improved by using the mutants molecules described: —binding to von Willebrand factor and therefore improved half-life of FVIII or circulating FVIIIa; —improved intrinsic stability of the molecule by stabilization of the A2 domain and therefore increase of its efficiency period; —binding to phospholipids derived from blood platelets, cell surfaces or circulating microparticles and therefore improved generation of FXa; —binding to FIXa and FX and therefore improved formation of FXa; —decreased binding of FVIII or FVIIIa to the molecules responsible of its catabolism such as for example low density Lipoprotein Receptor-related Protein (LRP), Low Density Lipoprotein Receptor (LDLR), Very Low Density Lipoprotein Receptor (VLDLR), megalin or any other receptor which might be identified and therefore improvement of the circulating FVIII half-life of; —decreased proteolysis of FVIII or FVIIIa by vascular proteases such as for example activated protein C, FXa, FIXa, and therefore increased of its efficiency period.

Preferably, the biologically active FVIII derivative is a FVIII consisting in a whole or partial deletion of the B domain. The human FVIII variant of the present invention is not a hybrid FVIII. It does not contain a substitution of the A2 or C2 domain or of a segment of at least 15 consecutive amino acids thereof by a FVIII domain of another species. In particular, segments of the A2 domain 373-540, 373-508, 445-508, 484-508, 404-508, 489-508 and/or 484-489 are not substituted by those of another species. In a particular embodiment, the polypeptide sequence of the variant differs from that of human FVIII such as described in SEQ ID No. 3 by 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 or 15 substitutions, preferably by 1, 2, 3, 4, 5, 6, 7 or 8 substitutions, without including an optional deletion or truncation. In a particular embodiment, the variant comprises a single substitution. In

another particular embodiment, the variant comprises a combination of 1 to 8 substitutions selected from a group according to the present invention.

“Inhibitory antibodies” or “inhibitors” refers to any antibody which recognizes or binds to FVIII and inhibits the biological activity thereof, in particular the procoagulant activity thereof. In particular, said antibodies can preferably recognize i) the C2 domain of the light chain (2181-2321); ii) the A2 domain of the heavy chain (484-509); or iii) the A3 domain (1694-2019). Examples of commercially available inhibitory antibodies comprise ESH-8 (strong inhibitor; recognized the region 2248/2285; 6300 BU/mg; anti-C2; America Diagnostica), GMA-8015 (anti-A2; Green Mountain), anti-C2 ESH-4 antibody (strong inhibitor; region 2303/2332; America Diagnostica), anti-C2 Bo2C11 antibody (Jacquemin et al., 1998, *Blood*, 92(2):496-506).

“Patients with inhibitors” are patients who have FVIII inhibitory antibodies in their serum. The recognition profile of said antibodies differs from a patient to another. An improved FVIII according to the present invention is a FVIII which at least partially avoids one or more types of inhibitory antibodies.

“Biologically active derivative of FVIII” refers to any protein or peptide derived from human FVIII which retains a procoagulant activity of FVIII. For example, such biologically active FVIII derivative may be a FVIII whose B domain (741-1648) has been partially or totally deleted (Toole et al., 1986, *Proc. Natl. Acad. Sci. USA*, 83 (16):5939-5942; Pittman, 1993, *Blood*, 81:2925-2935; Eaton et al., 1986, *Biochemistry*, 25 (26):8343-8347; Langer et al., 1988, *Behring Inst. Mitt.*, 82:16-25; Meulien et al., 1988, *Protein Eng.*, 2(4):301-6; and U.S. Pat. No. 4,868,112). Moreover, this term also refers to FVIII mutants with a stabilized A2 domain (WO 97/40145), FVIII mutants allowing a higher expression (Swaroop et al., 1997, *JBC*, 272:24121-24124), FVIII mutants having decreased antigenicity (Lollar, 1999, *Thromb. Haemost.*, 82:505-508), a FVIII reconstituted from separately expressed light and heavy chains (Oh et al., 1999, *Exp. Mol. Med.*, 31:95-100), FVIII mutants displaying decreased binding to FVIII catabolic associated receptors such as HSPG (heparan sulfate proteoglycans) and LRP (low density lipoprotein receptor related protein) (Ananyeva et al., 2001, *TCM*, 11:251-257), FVIII mutants displaying an improved specific activity (US2004/0249134). Also considered are FVIII variants in which FVIII segments are replaced by the corresponding segments of factor V (Marquette et al., 1995, *JBC*, 270:10297-10303, Oertel et al., 1996, *Thromb. Haemost.*, 75:36-44). Moreover, said term refers to any FVIII comprising one or more substitutions, deletions or insertions. For example, it comprises the variants described in the introduction of the present application, in particular those comprising point mutations. In particular, it comprises a FVIII less susceptible to cleavage by APC (activated protein C) comprising mutations of Arginines 336 and 562 and in the region comprised between the positions 2001-2020, as described in application WO 2006/027111. It further comprises a stabilized FVIII mutant in which one or more Cysteines have been introduced so as to create one or more disulfide bonds, for example between the A2 and A3 domains (WO02103024; Gale et Pellequer, 2003, *J Thromb Haemost.*, 1(9):1966-71). Patents JP2005112855 and RU2244556/RU2253475 respectively provide biologically stable and albumin-free compositions, allowing the stabilization of FVIII alone or in association with vWF. This term also refers to any FVIII having been modified by conjugation of a functional group, for example PEGylation, glycosylation (for

example US2005009148, US2003077752, etc.). Furthermore, the variant can comprise peptide bonds modified in order to resist to hydrolysis.

In particular, the variant has a decreased antigenicity towards inhibitory antibodies as compared to natural human FVIII and retains a procoagulant activity at least equal to 50% that of natural human FVIII. For example, one suitable assay is the one or two-stage clotting assay described in Rizza et al. (Rizza et al., 1982, Coagulation assay of Factor VIIIa and FIXa in Bloom ed. The Hemophilias. NY Churchill Livingston 1992). In a preferred embodiment, the variant retains a procoagulant activity equal to that of natural human FVIII. In a more preferred embodiment, the variant has a procoagulant activity higher than that of natural human FVIII.

The procoagulant activity of FVIII is determined by any method known to one skilled in the art. Preferably, said procoagulant activity is determined by chronometric assay or by chromogenic assay. Even more preferably, FVIII activity is determined by chronometric assay, for example as described by Von Clauss (A. Acta Haematologica, 1957, 17:237) or by chronometric assay such as described by Rosen (Scand. J. Haematol. 1984, 33 (Suppl 40):139-145).

The present invention relates to a nucleic acid coding for a human FVIII variant according to the invention. The present invention also relates to an expression cassette of a nucleic acid according to the invention. It further relates to a vector comprising a nucleic acid or an expression cassette according to the invention. The vector can be selected from a plasmid and a viral vector.

The nucleic acid can be DNA (cDNA or gDNA), RNA, or a mixture of the two. It can be in single stranded form or in duplex form or a mixture of the two. It can comprise modified nucleotides, comprising for example a modified bond, a modified purine or pyrimidine base, or a modified sugar. It can be prepared by any method known to one skilled in the art, including chemical synthesis, recombination, mutagenesis etc.

The expression cassette comprises all elements required for expression of the human FVIII variant according to the invention, in particular the elements required for transcription and translation in the host cell. The host cell can be prokaryotic or eukaryotic. In particular, the expression cassette comprises a promoter and a terminator, optionally an enhancer. The promoter can be prokaryotic or eukaryotic. Examples of preferred prokaryotic promoters include: LacI, LacZ, pLacT, ptac, pARA, pBAD, the RNA polymerase promoters of bacteriophage T3 or T7, the polyhedrin promoter, the PR or PL promoter of lambda phage. Examples of preferred eukaryotic promoters include: CMV early promoter, HSV thymidine kinase promoter, SV40 early or late promoter, mouse metallothionein-L promoter, and the LTR regions of some retroviruses. In general, to select a suitable promoter, one skilled in the art may advantageously consult Sambrook et al. work (1989) or techniques described by Fuller et al. (1996; Immunology in Current Protocols in Molecular Biology).

The present invention relates to a vector containing a nucleic acid or an expression cassette coding for a human FVIII variant according to the invention. The vector is preferably an expression vector, that is to say, it comprises the elements required for the expression of the variant in the host cell. The host cell can be a prokaryote, for example *E. coli*, or a eukaryote. The eukaryote can be a lower eukaryote such as a yeast (for example, *S. cerevisiae*) or fungus (for example from the genus *Aspergillus*) or a higher eukaryote such as an insect, mammalian or plant cell. The cell can be a mammalian cell, for example COS, CHO (U.S. Pat. Nos. 4,889,803 ; 5,047,335). In a particular embodiment, the cell is non-hu-

man and non-embryonic. The vector can be a plasmid, phage, phagemid, cosmid, virus, YAC, BAC, pTi plasmid from *Agrobacterium*, etc. The vector can preferably comprise one or more elements selected from the group consisting of a replication origin, a multiple cloning site and a selection gene. In a preferred embodiment, the vector is a plasmid. Examples of prokaryotic vectors include, but are not limited to, the following: pQE70, pQE60, pQE-9 (Qiagen), pbs, pD10, phagescript, psiX174, pbluescript SK, pbsks, pNH8A, pNH16A, pNH18A, pNH46A (Stratagene); ptrc99a, pKK223-3, pKK233-3, pDR540, pBR322, and pRIT5 (Pharmacia), pET (Novagen). Examples of eukaryotic vectors include, but are not limited to, the following: pWLNEO, pSV2CAT, pPICZ, pcDNA3.1 (+) Hyg (Invitrogen), pOG44, pXT1, pSG (Stratagene); pSVK3, pBPV, pCI-neo (Stratagene), pMSG, pSVL (Pharmacia); and pQE-30 (QLAexpress). Examples of viral vectors include, but are not limited to, adenoviruses, AAV, HSV, lentiviruses, etc. Preferably the expression vector is a plasmid or a viral vector.

The coding sequence for FVIII according to the present invention can comprise or not comprise the signal peptide. In the case where coding sequence does not comprise signal peptide, a methionine can optionally be added at the N-terminal end. Alternatively, a heterologous signal peptide can be introduced. Said heterologous signal peptide can be derived from a prokaryote such as *E. coli* or from a eukaryote, in particular from a mammalian, insect or yeast cell. Moreover, the nucleotide sequence can also comprise intron segments, particularly heterologous introns. Said intron segments can enable improved expression of the FVIII variant. Such constructs are described in application WO 2005/040213. For example, the nucleotide sequence can comprise modified sequence SEQ ID No. 5 so as to code for the FVIII variant comprising the substitution or substitutions according to the present invention.

The present invention relates to the use of a nucleic acid, an expression cassette or a vector according to the invention in order to transform or transfect a cell. The invention relates to a host cell comprising a nucleic acid, an expression cassette or a vector coding for a human FVIII variant and the use thereof to produce a recombinant human FVIII variant according to the invention. In a particular embodiment, the cell is non-human and non-embryonic. The invention also relates to a method for producing a recombinant human FVIII variant according to the invention comprising transforming or transfecting a cell by a nucleic acid, an expression cassette or a vector according to the invention; culturing the transformed/transfected cell; and collecting the human FVIII variant produced by the cell. In an alternative embodiment, the method for producing a recombinant human FVIII variant according to the invention comprises providing a cell comprising a nucleic acid, an expression cassette or a vector according to the invention; culturing the transfected/transformed cell; and collecting the human FVIII variant produced by the cell. In particular, the cell can be transformed/transfected in a transient or stable manner by the nucleic acid coding for the variant. Said nucleic acid can be contained in the cell in an episome form of or in chromosomal form. Method for producing recombinant proteins are well known to one skilled in the art. For example, one can mention the specific method described in WO0170968 for a production in an immortalized human cell line, WO2005/123928 for production in a plant, US2005/229261 for production in the milk of a transgenic animal, etc.

The present invention relates to pharmaceutical compositions comprising human FVIII variants according to the invention, and to the use of said FVIII variants for preparing

a medicament for the treatment of hemophilia A. Preferably, the hemophilia A is severe and moderate. Said treatment can be curative or preventive. In a particular embodiment, the treated patients are patients with inhibitors.

Thus, the FVIII variants according to the invention can be used in two major categories of hemophiliac patients: those who have developed FVIII inhibitory antibodies, thanks to their capacity to avoid said inhibitory antibodies, and those who have not yet developed such inhibitors, thanks to their lower risk of inducing the development of inhibitory antibodies as compared to the molecules currently used. Said FVIII variants will be usable by all patients with hemophilia A.

The present invention therefore relates to a pharmaceutical composition comprising a FVIII variant according to the invention. The pharmaceutical composition can further comprise compounds for stabilizing the mutant FVIII, for example serum albumin, vWF (von Willebrand factor) or a fragment thereof comprising the FVIII binding site, vitamin K-dependent coagulation factors, and polysaccharides such as sucrose. The present invention can also relate to a pharmaceutical composition comprising a nucleic acid coding for a FVIII mutant according to the invention, a vector or a host cell according to the invention. Such composition might be useful in the context of a gene therapy. The pharmaceutical composition can further comprise a pharmaceutically acceptable excipient or carrier. Such excipients and carriers are well known to one skilled in the art [Remington's Pharmaceutical Sciences, 18th edition, A. R. Gennaro, Ed., Mack Publishing Company (1990); Pharmaceutical Formulation Development of Peptides and Proteins, S. Frokjaer and L. Hovgaard, Eds., Taylor & Francis (2000); and Handbook of Pharmaceutical Excipients, 3rd edition, A. Kibbe, Ed., Pharmaceutical Press (2000)] and comprise physiological saline solutions and phosphate buffers. The FVIII variant according to the invention can also be formulated in a pharmaceutical composition with phospholipids or equivalents, for example in the form of liposomes, nanoparticles, etc. (WO2004/071420; WO2004/091723). The pharmaceutical composition can further comprise one or more other active ingredients.

The present invention also relates to a FVIII variant according to the invention as medicament. It further relates to a nucleic acid coding for a FVIII mutant, an expression cassette, a vector or a host cell according to the invention, as medicament.

The human FVIII variants of the invention can be used as replacement therapy in case of severe and moderate hemophilia A. The possibility of a continuously use with a lower risk of developing inhibitory antibodies is a major advantage over the different existing recombinant human or hybrid FVIII.

Said improved human FVIII variants are preferably intended for treating patients who have already developed inhibitors, but also for preventive treatment.

In addition, systematic administration of said FVIII might be encompass for a prophylactic treatment in any patient with hemophilia A. One might therefore imagine decreasing the risks of bleeding, for example during surgical procedures, or else preventing the development of inhibitors. The administration of said FVIII might also be considered in the case of an emergency treatment, for example during an accidental, pathological hemorrhage or caused by a surgical procedure.

The pharmaceutical compositions of the invention are suitable for oral, sublingual, subcutaneous, intramuscular, intravenous, topical, local, intratracheal, intranasal, transdermal, rectal, intraocular, intra-auricular administration, said active ingredient being able to be administered as a unit dose. Pref-

erably, the pharmaceutical compositions are suitable for intravenous, subcutaneous or intramuscular administration.

The dosages of the treatment can differ according to the severity of FVIII deficiency. Usually, the dosage is adjusted for frequency, period and units related to the severity and length of the bleeding episodes of the considered patient. FVIII is dosed so as to arrest bleeding, for example with standard clotting assays. An efficient dose of FVIII variant according to the invention can comprise, but is not limited to, between about 5 to 50 units per kg of body weight, preferably between 10 to 50, even more preferably between 20 to 40. The dosing frequency can be for example every 8 to 24 hours. The treatment duration can be for example from 1 to 10 days, or until bleeding stops. [See for example: Roberts, H. R., and M. R. Jones, "Hemophilia and Related Conditions—Congenital Deficiencies of Prothrombin (Factor II, Factor V, and Factors VII to XII), "Ch. 153, 1453-1474, 1460, in Hematology, Williams, W. J. et al., ed. (1990)].

The treatment can be in the form of a single intravenous injection or periodic or continuous administration over an extended period of time, as necessary. The treatment can also be administered by the subcutaneous or oral route with liposomes in one or more doses at different time intervals.

The present invention relates to the use of a human FVIII variant or a biologically active derivative thereof according to the invention for preparing a medicament for the treatment of coagulation disorders, in particular hemophilia A. The treatment can be curative or preventive. In a particular embodiment, the patient to be treated is a patient with inhibitors. The present invention also relates to a method for treating hemophilia A comprising administering a human FVIII variant or a biologically active derivative thereof according to the invention.

The present invention further relates to the use of a nucleic acid coding for a FVIII variant according to the invention for preparing a medicament for the treatment of coagulation disorders, in particular hemophilia A.

The FVIII variant of the invention can also be combined with another active compound. For example, the present invention also relates to the use of a FVIII variant according to the invention in combination with factor IXa for treating coagulation disorders, and in particular hemophilia A or B. Said combination is described in WO2004/103397.

The present invention further relates to the use of one or more human FVIII variants or a biologically active derivative thereof according to the invention for the diagnosis of inhibitor type in a patient with hemophilia A. In particular, the presence of inhibitory antibodies is assayed in serum samples or biological fluids (lymph, urine, etc.). Detection of inhibitory antibodies can be carried out by ELISA, immunodetection by electrophoretic blotting, radioimmunoassay, and FVIII activity assays (for example, clotting assay).

In fact, inventors have identified in wild-type human FVIII the positions specifically recognized by the inhibitors. Said positions can be used individually, combined within a same domain, or combined between the A2 and C2 domains, so as to reveal the type(s) of inhibitory antibodies present in a hemophiliac. In fact, the need to diagnose inhibitory antibodies is crucial. The titration of said inhibitors is a prerequisite prior to any replacement therapy. The inventors therefore propose to use of the present findings to diagnose inhibitory antibodies. A Bethesda assay (assay of inhibitor titer) in a hemophiliac patient can be carried out before and after passage on ELISA where the capture antigen corresponds to the FVIII variants of the present invention taken separately or combined. The inhibitor titer will significantly decrease for the control carried out with wild-type FVIII. The variant or

variants combination for which the inhibitor titer remains unchanged is used as treatment for the hemophiliac patient with inhibitors. This diagnosis therefore renders possible to control and target the delivery of the human FVIII variant according to the invention.

Thus, the present invention relates to a method for treatment comprising:

a recognition test of inhibitory antibodies contained in a serum sample of patient on one or more FVIII variants according to the invention;

selection of the FVIII mutant or mutants which are not recognized by said inhibitory antibodies; and

administration of one or more FVIII mutants selected from b).

In a preferred manner, the recognition test between the patient's sample and the FVIII variant(s) according to the invention is carried out by a Bethesda assay. As a control, a recognition test is preferably carried out on wild-type FVIII.

The present invention relates to a diagnostic kit comprising one or more FVIII variants according to the invention.

The present invention also relates to the use of one or more human FVIII variants or a biologically active derivative thereof according to the invention for preparing a medication for the treatment of hemophilia A in patients with inhibitors whose serum does not contain antibodies recognizing said human FVIII variant(s) or a biologically active derivative thereof.

All references cited herein are included by reference in the present application. Other features and advantages of the invention will become apparent in the following examples which are provided for purposes of illustration and not by way of limitation.

## EXAMPLES

### Example 1

#### Molecular Biology

FVIII complementary DNA containing two truncated introns of factor IX at position 1 and 13 (5012 bp) (SEQ ID No. 4) was cloned between the NotI and XhoI restriction sites in a vector (pcDNA3.1 GS, Invitrogen) allowing expression of the protein in mammalian cells. The pcDNA/FVIII construct corresponded to a 10,439 bp plasmid. This gene comprises the five functional domains A1, A2, A3, C1 and C2 essential for FVIII activity. As it has previously been shown that the B domain does not play any predominant role in the procoagulant function of FVIII, the inventors chose to produce FVIII with a deletion of this domain. The regions coding for the A1 and A2 domains each contain an intron. Insertion of these two intron regions among the coding exons significantly improves the expression of human FVIII. The protein sequence encoded by this gene is given in SEQ ID No. 5.

The mutagenesis strategy consisted in systematically generating all the single Alanine mutants in the targeted domains of FVIII, i.e., A2, A3 and C2. Said mutants were generated by the Massive Mutagenesis® method described in US2004/0048268.

As mentioned earlier, it has been shown that domains A2, C2 and A3 are the preferential targets of FVIII recognition by inhibitory antibodies. Each amino acid in these functional domains was substituted by an Alanine, apart from the intron segment of the A2 domain. A series of 795 oligonucleotides (32-mers) was designed and produced so as to introduce an Alanine mutation at positions i) 376 to 719 [A2]; ii) 2173 to 2325 [C2]; iii) 1691 to 2025 [A3]. The numbering system for

the mutations of human FVIII used in the invention is that defined by Wood et al. (Nature, 1984, 312:330-337). After site-directed mutagenesis, the inventors performed two successive sequencings to check that each mutant of the library contained the Alanine mutation at the considered position. This collection of Alanine mutants in the C2, A2 and A3 domains of FVIII is the first comprehensive site-directed mutants library ever carried out for this molecule.

### Example 2

#### Expression of Human FVIII Alanine Mutants in COS-7 Mammalian Cells

FVIII is usually expressed in mammalian cells (Toole et al., 1984, Nature, 312:342-347; Gitschier et al., 1984, Nature, 312:326-330; Wood et al., 1984, Nature, 312:330-337; Vehar et al., 1984, Nature, 312:337-342; WO8704187; WO 8808035; WO8803558; U.S. Pat. No. 4,757,006).

In order to transfect COS-7 cells with the native or mutated pcDNA/FVIII constructs, said cells were trypsinized when they reached 90% confluence. The COS-7 cells were reseeded at a 1/4 ratio (that is, in order to obtain approximately 25% confluence once they adhered to the surface). Transient transfection of COS-7 cells was carried out in 90 mm culture plates (6 ml per well) when cells reached 70-80% confluence. Transfection was carried out with approximately 6 µg DNA for a volume of 18 µl FuGENE-6 (Roche, Meylan, France).

Prior to transfection, FuGENE-6 was diluted in serum-free IMDM medium and incubated at room temperature for 5 min. The FuGENE-6/DNA mixture was left at room temperature for 15 min then deposited dropwise on the cells in complete medium. A first supernatant containing FVIII was collected 24 h after transfection; 6 ml of fresh medium were then placed on the cells. The culture supernatant was collected 48 h later (6 ml), aliquoted and stored at -20° C. pending the clotting assay (chromogenic). The mean level of expression of wild-type FVIII was estimated by ELISA (Stago commercial ELISA kit) and was comprised between 20 and 60 ng/ml.

All cell culture reagents were from Invitrogen. COS-7 cells (African green monkey SV40 transformed kidney cells) were grown in standard culture conditions (37° C. in a humid 5% CO<sub>2</sub> atmosphere) using Iscove's Modified Dulbecco's Medium (IMDM). IMDM was supplemented with an L-glutamine analog (glutamax), decomplexed fetal calf serum (10% final concentration) and antibiotics (penicillin 40 U/ml and streptomycin 0.1 mg/ml).

### Example 3

#### Primary Screen: Functional Analysis of Human FVIII Alanine Mutants

The primary screen correlates to raw coagulant activity determination (FIG. 1) obtained in a same volume of COS-7 cell culture supernatant. Two different assays of clotting activity determination were used in the primary screen, the chronometric assay and the chromogenic assay.

Chronometric activity was measured following incubation of a dilution of the FVIII molecules to be tested in imidazole buffer in the presence of FVIII-deficient plasma (Stago). Clotting was initiated by addition of calcium and the time to clot formation was determined on a MDA-II apparatus (BioMérieux, Marcy-l'Étoile). The coagulant activity of the 795 Alanine mutants was measured by chronometric assay on a robotic platform of the National Hemophilia Treatment

Center (Hospices Civils de Lyon). The chromometric activity of all the Alanine mutants was compared to the activity of a wild-type FVIII used as internal standard for each transfection. Results of these determinations of raw activity relative to that of non-mutated FVIII distinguished two categories of mutants: i) mutants having retained at least 50% of wild-type FVIII activity; ii) mutants having less than 50% of wild-type FVIII activity. FIG. 2 shows the coagulant activity of 359 over 795 Alanine mutants analyzed. These data represent a functional mapping of each of these FVIII residues for coagulant activity; a coagulant activity suppressed by an Alanine mutation indicates that the considered residue is essential for FVIII coagulant activity.

158 mutants having retained more than 50% of raw non-mutated FVIII activity were selected by this chromometric assay for secondary screen. Their activities were first confirmed by the second clotting assay, the chromogenic assay mentioned above. This assay was also performed on the robotic platform of the National Hemophilia Treatment Center (Hospices Civils de Lyon). The chromogenic activity of the 158 selected Alanine mutants was carried out with the Coamatic Factor VIII kit (Chromogenix, Instrumentation Laboratory, Milan, Italy) according to the supplier's instructions. Briefly, culture supernatants (50  $\mu$ l) were diluted in the dilution buffer provided and preincubated at 37° C. for 4 min. The reaction medium (50  $\mu$ l), preheated at 37° C., was then added for 4 min, after which 50  $\mu$ l of development medium at 37° C. were added. The formation of product over time was measured immediately on a spectrophotometer at 405 nm after shaking the microtiter plate. Product formation is expressed as mUOD/min. When values were greater than 200 mUOD/min, the assay was repeated using a higher dilution.

Table 1 shows the activities of the 158 mutants which retained more than 50% of non-mutated FVIII activity. Said 158 mutants were selected for the secondary screening.

#### Example 4

##### Secondary Screen: Evaluation of Loss of Antigenicity Towards Human FVIII Inhibitory Antibodies

The secondary screen correlates to an assay similar to the Bethesda assay, carried out as described below on the 158 mutants selected following the primary screening; said assay comprises a step of contacting a inhibitory serum (or antibody) with a FVIII molecule to be tested or a reference standard and a step of measuring FVIII coagulant activity by chromometric assay.

Culture supernatants obtained after 48 h of contact with COS cells transfected by different FVIII constructs were used. Said supernatants were produced in complete medium [(IMDM, Invitrogen), 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin]. Supernatants were diluted in fresh complete medium to obtain a final chromometric activity comprised in the range of about 10-20% (1 FVIII unit=100% activity=200 ng/ml). The culture supernatant diluted or not (140  $\mu$ l) was added to 150  $\mu$ l of FVIII-depleted human plasma (Stago, Asnières, France). An antibody dilution (10  $\mu$ l) was then added to the mix. These antibodies are IgG fractions purified on protein A—from hemophiliac patients with inhibitors. An IgG fraction from a non-hemophiliac control was similarly obtained. Bethesda inhibitor titers were identical to the inhibitory activity from the plasma. The purification protocol therefore did not affect the inhibitory activity of the antibodies. The antibodies were first diluted in fresh complete medium, the measurement

being carried out either with a fixed antibody dilution or with serial dilutions. The fixed antibody concentration which was used was that which produced 50% inhibition of a recombinant FVIII standard solution with 12.5% activity. Samples were incubated in a 37° C. water-bath for 1 h 30. Coagulant activity was then determined on a MDA-II apparatus (BioMérieux, Marcy-l'Etoile) and compared to that of a standard curve established from an identical FVIII stably produced in the CHO cell line. Results are expressed as a percentage which represents the abolition to inhibition of coagulant activity of a given mutant by inhibitory antibodies from a patient's serum. Said percentage was calculated as shown in FIG. 5 for the FVIII mutant E518A. Abolition to inhibition expressed is a percentage= $-(b-a)/a \times 100$ ; where "a" is the percentage residual activity of the WT (serum+IgG/serum-IgG) and "b" is the percentage residual activity of the mutant (serum+IgG/serum-IgG).

Table 2 shows for 30 single mutants the percentages of abolition to inhibition for sera from five hemophiliac patients. Said mutants were selected in the secondary screen of the 158 mutants selected in the primary screen. Several mutants show a high percentage of abolition to inhibition with certain sera, such as mutant 2316 for sera TD and SL, mutant 2294 for serum GC, mutant 403 for serum FS and mutant 2275 for serum PR.

Patients' sera were selected for their high Bethesda titers (greater than 10 BU) and their different inhibitor profiles. These patients can no longer be treated with FVIII injections and need bypassing agents. Thus, obtaining FVIII Alanine mutants which abolish, even partially, the inhibition of FVIII activity by the inhibitory antibodies of one of these patients, is a major step forward to the future approaches of treating hemophiliac patients with inhibitors. The different data obtained on a large number of mutants as well as the different sera tested will make it possible to create combinations of mutations leading to an improved FVIII which can avoid a majority of inhibitory antibodies while retaining its procoagulant activity.

The reproducibility of FVIII expression level related to transfections was controlled by following the specific activity of wild-type FVIII. Indeed, specific activities calculated from antigen determinations (Stago commercial ELISA kit) were identical for wild-type FVIII produced in different transfections. Likewise, antigen concentrations were determined for mutants having retained at least 50% of wild-type FVIII activity and their specific activity was determinate throw. Specific activity corresponds to raw activity measured in the chromogenic assay (mUOD/min) relative to protein concentration (ng/ml) obtained with an ELISA kit (Stago FVIII kit). Table 3 shows comparative data of raw and specific activities of 30 mutants selected in the secondary screen.

The eight FVIII Alanine mutants 2175, 2199, 2200, 2215, 2251, 2252, 2278 and 2316 displayed a far above average capacity to be secreted in the COS cell production medium used in the scope of the present invention. FIG. 3 depicts the data obtained for these eight mutants. Raw coagulant activity of these mutants was determined by chromogenic assay. Their concentration was approximately two to four times higher than that of wild-type FVIII. This property is interesting for producing recombinant FVIII and might make it possible to lower production costs of a new generation FVIII. Also, it might be advantageous in a gene therapy for hemophiliac patients. Moreover, these mutations which confer a greater capacity to be secreted may be of major interest in combination with mutations conferring abolition to inhibi-

tion by inhibitory antibodies, by allowing, for example, to compensate an optional relative loss of secretion of said less antigenic mutants.

The 15 mutants 2177, 2183, 2186, 2191, 2196, 2204, 2205, 2206, 2213, 2217, 2235, 2258, 2264, 2268 and 2269 displayed far higher specific activity than wild-type FVIII, while maintaining a high production level, around to that of wild-type FVIII (concentration greater than 10 ng/ml). The specific activities of these 15 mutants are given in FIG. 4. Raw coagulant activity of these mutants was determined by chromogenic assay. This property is interesting because it would allow smaller or less frequent doses of FVIII to be injected in patients. Moreover, these mutations which confer a higher specific activity might be of major interest in combination with mutations conferring abolition to inhibition by inhibitory antibodies, by allowing to compensate an optional relative loss of activity of said less antigenic mutants.

#### Example 5

##### Selection and Combination of the Best Single Mutants Selected in the Secondary Screen

Among the 30 single mutants selected in the secondary screen, eight were chosen in order to combine their respective mutations, to obtain a cumulative/additive effect of remarkable properties of each. The selection criteria for these mutants were complex and considered the following parameters:

- at least 25% abolition to inhibition for at least one of the test sera from hemophilic patients with inhibitors;
- raw coagulant activity at least 100% relative to non-mutated FVIII; and
- reproducibly good level of expression.

The eight selected mutants were mutants 409, 462, 507 and 629 in the A2 domain and mutants 2289, 2294, 2312 and 2316 in the C2 domain. As noted earlier, the selection criterion considered of a high specific activity (coagulant activity relative to expression level), as shown in Table 3. This specific activity level had to be constant in the different experiments.

The 28 double mutants resulting from the combination of the eight single mutations 409, 462, 507, 629, 2289, 2294, 2312 and 2316 (six A2 double mutants+six C2 double mutants+sixteen A2-C2 double mutants presented in Table 4) were constructed by mutagenesis methods known to one skilled in the art. These mutants were transiently expressed in COS-7 mammalian cells as described in Example 2. Their expression level and their activity level were determined as described in the previous examples, respectively by ELISA and chromogenic assay (mUOD/min). These 28 mutants were then assessed for their abolition to inhibition by antibodies from hemophilic patients. The A2 double mutants displayed a significant abolition to inhibition for one or all of the antibodies from the patients' sera, whereas the combinations containing C2 domain mutations (six C2 double mutants+sixteen A2-C2 double mutants) displayed an insignificant or null abolition to inhibition.

Table 5 shows the specific activities of the six A2 double mutants and their percentage of abolition to inhibition by sera from four hemophilic patients TD, GC, SL and PR calculated as in Example 4. Especially preferred double mutants significantly abolished antibodies from a minimum of three over the four patients. This illustrates the cumulative effect of the four single mutations in the A2 domain. The choice was therefore based on the combination of the four mutations 409,

507, 462 and 629. Triple mutants and the quadruple mutant comprising these four mutations 409, 507, 462 and 629 were also constructed.

#### Example 6

##### Construction and Characterization of a Quadruple Mutant (FVIII-4A2)

The quadruple mutant derived from the combination of the four selected A2 mutations 409, 462, 507, 629 was constructed by a classical mutagenesis method known to one skilled in the art. The quadruple mutant was produced in a CHO cell line obtained as described in Example 9. This mutant was also characterized for its abolition to inhibition by antibodies from five hemophilic patients FS, TD, GC, PR and SL. Residual activity determined after incubation with an inhibitory antibody is divided by residual activity remaining after incubation with a non-immune antibody. The percentage of residual activity was thus determined and is presented in the graphs of FIG. 6. These graphs illustrate the residual activity of FVIII-4A2 after contact with different dilutions of antibodies from the different patients with inhibitors. It clearly appears that the FVIII-4A2 mutant retained a much higher chromometric activity after incubation with the inhibitory antibodies. Accordingly, the increases in residual activity for the highest inhibitory antibodies concentrations ranged from 230 to 450%, said percentage of residual activity depending on both the source of the inhibitory antibody and the concentration used.

To determine whether direct binding of the antibodies to FVIII-4A2 was modified, three additional antibodies were used instead of the patients' sera according to the same protocol as above: an anti-A2 domain antibody (GMA012, Green Mountain Antibodies), an anti-C2 domain antibody (ESH4, American Diagnostica) and a rabbit polyclonal antibody, purified from the same protocol used for the patients' antibodies. The results of these controls are shown in FIG. 7 for the two anti-A2 domain antibodies, the rabbit polyclonal antibody and GMA012. Clearly, the mutations in the A2 domain of FVIII-4A2 allowed FVIII-4A2 to avoid the anti-A2 domain antibody, GMA012 and the rabbit polyclonal antibody (shown). On the other hand, no significant differences in inhibition of FVIII-4A2 versus wild-type FVIII were seen for ESH4 (data not shown). These findings correlate the abolition to inhibition data, showing on one hand that introduction of mutations in the A2 domain allow to avoid patients' antibodies and on the other hand that the C2 domain of FVIII-4A2 is undamaged since recognition is similar to that of wild-type FVIII. This latter point is important for FVIII-4A2 activity because it is the C2 domain which is responsible for interactions with von Willebrand factor and with the cofactors required for full FVIII activity (calcium and phospholipid binding).

#### Example 7

##### Characterization of the FVIII 4A2 Mutant

a) ELISA  
FVIII-4A2 was produced in the same CHO cell line as wild-type FVIII according to the protocol described in Example 9. It was purified by the same protocol (also described in Example 9) and was therefore compared to FVIII in functional analyses. FVIII-4A2 concentrations were determined with an ELISA kit (see protocol below). Additional controls were performed using a panel of monoclonal anti-



bodies to check that the introduced mutations did not alter the quantification of mutant FVIII with this kit. Thereby, it was shown that similar concentrations of wild-type FVIII and FVIII-4A2 were identically recognized by antibody ESH-4 directed against the light chain C2 domain. In agreement with the abolition to inhibition data, there was a large decrease in recognition of FVIII-4A2 by the GMA012 antibody in comparison with wild-type FVIII. These data are presented in FIG. 8.

The protocol of the ELISA assays for these experiments is described below:

Reagent was diluted at least five-fold in 50 mM CAPS pH 9.0 and incubated overnight at 4° C. to coat the interest product on the support of the ELISA plate (Nunc Maxisorb). Wells were then washed twice with TBS-T buffer (50 mM Tris-HCl pH 8.0, 100 mM NaCl, 5 mM MgCl<sub>2</sub>, 0.01% Tween 20, 0.05% BSA), then blocked for 1 h with TBS-3% BSA (50 mM Tris-HCl pH 8.0, 100 mM NaCl, 5 mM MgCl<sub>2</sub>, 0.01% Tween-20, 3% BSA). Reagent binding with the one coated on the plate was then diluted in TBS-3% BSA, incubated at room temperature for 1 h 30, then washed three times in TBS-T. Primary and secondary antibodies conjugated to horse radish peroxidase (HRP) were diluted in TBS-3% and respectively added for 1 h 30 at room temperature. Secondary antibodies were diluted 2000-fold. Between two antibody incubations, plates were washed three times with TBS-T, then washed again before addition of the substrate, a mixture of OPD/urea (Sigma). The enzymatic reaction was stopped by adding 2.5M H<sub>2</sub>SO<sub>4</sub>. Optical density was read at 490 nm.

#### b) Measurement of Specific Activity

Specific activity of the FVIII-4A2 mutant was determined by dividing chromogenic activity by concentration. These specific activities were compared with those of the wild-type. The chromogenic activity of wild-type FVIII was about 15±1 ODU/min·µg and that of FVIII-4A2 was about 27±1 ODU/min·µg, that is, a higher activity.

#### c) Activation by Thrombin

Wild-type FVIII and FVIII-4A2 (0.125 U or 25 ng) were diluted in 40 mM HEPES buffer, 100 mM NaCl, 5 mM CaCl<sub>2</sub> containing 10 µM of an 80:20 mixture of Phosphatidylcholine:Phosphatidylserine and 0.1 mg/ml BSA, then incubated at 37° C. for 5 min. Thrombin (0.05 U) was added and its action determined at different time. At each time, an aliquot was removed and incubated with a mixture of hirudin (0.5 U), factor IXa (50 nM) and factor X (200 nM) diluted in the same buffer, in order to generate FXa. The FXa substrate pNAPEP-25 was immediately added and formation of the chromogenic product was measured at 405 nm. The initial rate was determined and the amount of FXa formed per minute was calculated.

Wild-type FVIII and FVIII-4A2 displayed an identical thrombin response profile, with a rapid increase in FVIII activity, reaching the peak at 1-2 min after addition of thrombin, followed by a rapid decrease of said activity with a half-life of approximately 2-3 min. The results shown in FIG. 9 indicate that FVIII-4A2 is identically recognized by thrombin as wild-type FVIII with a relative decrease of activity which might be caused by one of the four mutations.

#### d) Dissociation of the A2 Domain

Wild-type FVIII and FVIII-4A2 were activated as described above for 1 min. Hirudin was then added and FVIIIa was left at 37° C. for different time periods. Aliquots were removed at said time and incubated with a mixture of phospholipids, FIXa and FX. FXa was allowed to form for 5 min, then Stop buffer was added (Iris 50 mM pH 8.8, 475 mM NaCl, 9 mM EDTA). The amount of FXa formed was determined as above.

FVIIIa was incubated for different times before determining its residual activity. The loss of activity over time corresponds to dissociation of the A2 domain. The loss of activity profile of wild-type FVIII and FVIII-4A2 was similar but the respective kinetics differed. Indeed, wild-type FVIII had a half-life of 3 min while that of FVIII-4A2 was 11 min. This increased stability may explain the higher specific activity observed in the chromogenic assay. In this test, FVIIIa was incubated for 4 min before adding the substrate. Wild-type FVIII thus lost its activity faster than FVIII-4A2 during this test. The results are shown in FIG. 10.

### Example 8

#### Construction and Characterization of FVIII-3A2 Mutants

Four triple FVIII-3A2 mutants were constructed: FVIII-3A2 (409-462-507), FVIII-3A2 (462-507-629), FVIII-3A2 (409-462-629), FVIII-3A2 (409-507-629).

#### FVIII-3A2 (409-462-507) Specific Activity Determination

The specific activity of the FVIII-3A2 mutant (409-462-507) was determined by dividing chromogenic or chromogenic activity by concentration. These specific activities were compared with that of wild-type FVIII. The chromogenic activity of FVIII-3A2 (409-462-507) was 98% of the chromogenic activity of wild-type FVIII. These results indicate that the absence of mutation at position 629 in FVIII-3A2 yielded a higher coagulant activity than for FVIII-4A2.

#### FVIII-3A2 (409-462-507) Abolition to Inhibition

This mutant was also analyzed for its abolition to inhibition by antibodies from the four hemophiliac patients FS, TD, GC and SL. Residual activity determined after incubation with an inhibitory antibody was divided by the activity remaining after incubation with a non-immune antibody. The percentage of residual activity was thus determined and is presented in FIG. 11 curves. These curves illustrate the residual activity of FVIII-3A2 (409-462-507) after contact with different dilutions of antibodies from the different patients with inhibitors. It clearly appears that the use of the FVIII-3A2 mutant (409-462-507) enable to retain a much higher chronometric activity after incubation with inhibitory antibodies. The combination of mutations 409-462-507 therefore yields a greater abolition to inhibition resulting in an increase in residual activity. This percentage of residual activity depends on both the source of inhibitory antibody and the concentration used.

### Example 9

#### Production of a CHO Cell Line Expressing FVIII-4A2 and Purification/ Production of FVIII

#### Production of the CHO Cell Line

A CHO cell line (ECACC 85050302) expressing FVIII was generated as described in Plantier et al. (Thrombosis and Haemostasis 2001; 86 p. 596). Briefly, cells were maintained at 37° C. in a humid 5% CO<sub>2</sub> atmosphere. Cells were grown in IMDM medium supplemented with 10% fetal calf serum and 1% penicillin-streptomycin. Cells (7×10<sup>6</sup>) were trypsinized and resuspended in PBS, then subjected to electroporation in presence of a cDNA of interest (7 µg). Cells were then reseeded in the presence of geneticin (0.6 mg/ml). Individual clones were selected, subcultured and amplified. Cells' ability to synthesize FVIII was determined by measuring the chromogenic activity of the culture medium. The best producer clones were amplified and grown in triple flasks. Production took place over 5 days during which cells were

incubated in complete medium during the day, washed three times, then incubated overnight in IMDM medium containing 1% BSA instead of serum. The BSA-containing medium was collected, centrifuged at 2500 rpm for 10 min at 4° C. and stored at -30° C. Cells were put back into complete medium during the day.

Purification and Production of FVIII Mutants (FVIII-3A2 and FVIII-4A2)

The purification protocol was based on the technique described by Jenkins et al. (Blood, 2004). The culture medium was thawed and 40% (m/V) (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> was added. The medium was shaken overnight at 4° C., then centrifuged at 14,000 rpm for 30 min at 4° C. The pellet was resuspended 1 in 10 by volume in 20 mM MES pH 6.0, 100 mM NaCl, 5

mM CaCl<sub>2</sub>, 0.01% Tween-20 buffer and dialyzed overnight against a similar buffer but containing 200 mM NaCl. Dialysate was centrifuged at 13,000 rpm for 10 min at room temperature, then loaded at 2 ml/min on a FLPC Sepharose FF column. The column was previously equilibrated with the same buffer. FVIII was eluted in a 0.2 to 1 M NaCl gradient. Fractions containing the highest chromogenic activity were pooled and dialyzed against 50 mM HEPES pH 7.4, 100 mM NaCl, 5 mM NaCl and 0.01% Tween-20 buffer. Dialysate was aliquoted and stored at -80° C. The quality of the protein was assessed after migration on SDS-PAGE 10% acrylamide by silver nitrate staining and by immunoblot. FVIII concentration was determined by the Asserachrom FVIII:Ag kit (Stago, Asnieres, France).

## SEQUENCE LISTING

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                                     1
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      5              10              15
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Tyr Met Gln Ser Asp Leu Gly Glu Leu Pro Val Asp Ala Arg Phe Pro
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cct aga gtg cca aaa tct ttt cca ttc aac acc tca gtc gtg tac aaa      369
Pro Arg Val Pro Lys Ser Phe Pro Phe Asn Thr Ser Val Val Tyr Lys
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Ile	Leu	Gly	Pro	Leu	Leu	Tyr	Gly	Glu	Val	Gly	Asp	Thr	Leu	Leu	Ile	
		470					475						480			
ata	ttt	aag	aat	caa	gca	agc	aga	cca	tat	aac	atc	tac	cct	cac	gga	1665
Ile	Phe	Lys	Asn	Gln	Ala	Ser	Arg	Pro	Tyr	Asn	Ile	Tyr	Pro	His	Gly	
		485					490						495			
atc	act	gat	gtc	cgt	cct	ttg	tat	tca	agg	aga	tta	cca	aaa	ggt	gta	1713
Ile	Thr	Asp	Val	Arg	Pro	Leu	Tyr	Ser	Arg	Arg	Leu	Pro	Lys	Gly	Val	
	500					505					510					
aaa	cat	ttg	aag	gat	ttt	cca	att	ctg	cca	gga	gaa	ata	ttc	aaa	tat	1761
Lys	His	Leu	Lys	Asp	Phe	Pro	Ile	Leu	Pro	Gly	Glu	Ile	Phe	Lys	Tyr	
515					520					525					530	
aaa	tgg	aca	gtg	act	gta	gaa	gat	ggg	cca	act	aaa	tca	gat	cct	cgg	1809
Lys	Trp	Thr	Val	Thr	Val	Glu	Asp	Gly	Pro	Thr	Lys	Ser	Asp	Pro	Arg	
			535					540						545		
tgc	ctg	acc	cgc	tat	tac	tct	agt	ttc	ggt	aat	atg	gag	aga	gat	cta	1857
Cys	Leu	Thr	Arg	Tyr	Tyr	Ser	Ser	Phe	Val	Asn	Met	Glu	Arg	Asp	Leu	
			550					555						560		
gct	tca	gga	ctc	att	ggc	cct	ctc	ctc	atc	tgc	tac	aaa	gaa	tct	gta	1905
Ala	Ser	Gly	Leu	Ile	Gly	Pro	Leu	Leu	Ile	Cys	Tyr	Lys	Glu	Ser	Val	
		565					570						575			
gat	caa	aga	gga	aac	cag	ata	atg	tca	gac	aag	agg	aat	gtc	atc	ctg	1953
Asp	Gln	Arg	Gly	Asn	Gln	Ile	Met	Ser	Asp	Lys	Arg	Asn	Val	Ile	Leu	
	580					585					590					
ttt	tct	gta	ttt	gat	gag	aac	cga	agc	tgg	tac	ctc	aca	gag	aat	ata	2001
Phe	Ser	Val	Phe	Asp	Glu	Asn	Arg	Ser	Trp	Tyr	Leu	Thr	Glu	Asn	Ile	
595					600					605					610	
caa	cgc	ttt	ctc	ccc	aat	cca	gct	gga	gtg	cag	ctt	gag	gat	cca	gag	2049
Gln	Arg	Phe	Leu	Pro	Asn	Pro	Ala	Gly	Val	Gln	Leu	Glu	Asp	Pro	Glu	
			615					620						625		
ttc	caa	gcc	tcc	aac	atc	atg	cac	agc	atc	aat	ggc	tat	ggt	ttt	gat	2097
Phe	Gln	Ala	Ser	Asn	Ile	Met	His	Ser	Ile	Asn	Gly	Tyr	Val	Phe	Asp	
		630						635						640		
agt	ttg	cag	ttg	tca	ggt	tgt	ttg	cat	gag	gtg	gca	tac	tgg	tac	att	2145
Ser	Leu	Gln	Leu	Ser	Val	Cys	Leu	His	Glu	Val	Ala	Tyr	Trp	Tyr	Ile	
		645					650						655			
cta	agc	att	gga	gca	cag	act	gac	ttc	ctt	tct	gtc	ttc	ttc	tct	gga	2193
Leu	Ser	Ile	Gly	Ala	Gln	Thr	Asp	Phe	Leu	Ser	Val	Phe	Phe	Ser	Gly	
	660					665					670					
tat	acc	ttc	aaa	cac	aaa	atg	gtc	tat	gaa	gac	aca	ctc	acc	cta	ttc	2241
Tyr	Thr	Phe	Lys	His	Lys	Met	Val	Tyr	Glu	Asp	Thr	Leu	Thr	Leu	Phe	
675					680					685					690	
cca	ttc	tca	gga	gaa	act	gtc	ttc	atg	tgc	atg	gaa	aac	cca	ggt	cta	2289
Pro	Phe	Ser	Gly	Glu	Thr	Val	Phe	Met	Ser	Met	Glu	Asn	Pro	Gly	Leu	
			695					700						705		
tgg	att	ctg	ggg	tgc	cac	aac	tca	gac	ttt	cgg	aac	aga	ggc	atg	acc	2337
Trp	Ile	Leu	Gly	Cys	His	Asn	Ser	Asp	Phe	Arg	Asn	Arg	Gly	Met	Thr	
			710					715						720		
gcc	tta	ctg	aag	ggt	tct	agt	tgt	gac	aag	aac	act	ggt	gat	tat	tac	2385
Ala	Leu	Leu	Lys	Val	Ser	Ser	Cys	Asp	Lys	Asn	Thr	Gly	Asp	Tyr	Tyr	
		725					730							735		
gag	gac	agt	tat	gaa	gat	att	tca	gca	tac	ttg	ctg	agt	aaa	aac	aat	2433
Glu	Asp	Ser	Tyr	Glu	Asp	Ile	Ser	Ala	Tyr	Leu	Leu	Ser	Lys	Asn	Asn	
	740					745					750					
gcc	att	gaa	cca	aga	agc	ttc	tcc	cag	aat	tca	aga	cac	cct	agc	act	2481
Ala	Ile	Glu	Pro	Arg	Ser	Phe	Ser	Gln	Asn	Ser	Arg	His	Pro	Ser	Thr	
	755				760					765					770	
agg	caa	aag	caa	ttt	aat	gcc	acc	aca	att	cca	gaa	aat	gac	ata	gag	2529
Arg	Gln	Lys	Gln	Phe	Asn	Ala	Thr	Thr	Ile	Pro	Glu	Asn	Asp	Ile	Glu	

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775	780	785	
aag act gac cct tgg ttt gca cac aga aca cct atg cct aaa ata caa Lys Thr Asp Pro Trp Phe Ala His Arg Thr Pro Met Pro Lys Ile Gln 790 795 800			2577
aat gtc tcc tct agt gat ttg ttg atg ctc ttg cga cag agt cct act Asn Val Ser Ser Ser Asp Leu Leu Met Leu Leu Arg Gln Ser Pro Thr 805 810 815			2625
cca cat ggg cta tcc tta tct gat ctc caa gaa gcc aaa tat gag act Pro His Gly Leu Ser Leu Ser Asp Leu Gln Glu Ala Lys Tyr Glu Thr 820 825 830			2673
ttt tct gat gat cca tca cct gga gca ata gac agt aat aac agc ctg Phe Ser Asp Asp Pro Ser Pro Gly Ala Ile Asp Ser Asn Asn Ser Leu 835 840 845 850			2721
tct gaa atg aca cac ttc agg cca cag ctc cat cac agt ggg gac atg Ser Glu Met Thr His Phe Arg Pro Gln Leu His His Ser Gly Asp Met 855 860 865			2769
gta ttt acc cct gag tca ggc ctc caa tta aga tta aat gag aaa ctg Val Phe Thr Pro Glu Ser Gly Leu Gln Leu Arg Leu Asn Glu Lys Leu 870 875 880			2817
ggg aca act gca gca aca gag ttg aag aaa ctt gat ttc aaa gtt tct Gly Thr Thr Ala Ala Thr Glu Leu Lys Lys Leu Asp Phe Lys Val Ser 885 890 895			2865
agt aca tca aat aat ctg att tca aca att cca tca gac aat ttg gca Ser Thr Ser Asn Asn Leu Ile Ser Thr Ile Pro Ser Asp Asn Leu Ala 900 905 910			2913
gca ggt act gat aat aca agt tcc tta gga ccc cca agt atg cca gtt Ala Gly Thr Asp Asn Thr Ser Ser Leu Gly Pro Pro Ser Met Pro Val 915 920 925 930			2961
cat tat gat agt caa tta gat acc act cta ttt ggc aaa aag tca tct His Tyr Asp Ser Gln Leu Asp Thr Thr Leu Phe Gly Lys Lys Ser Ser 935 940 945			3009
ccc ctt act gag tct ggt gga cct ctg agc ttg agt gaa gaa aat aat Pro Leu Thr Glu Ser Gly Gly Pro Leu Ser Leu Ser Glu Glu Asn Asn 950 955 960			3057
gat tca aag ttg tta gaa tca ggt tta atg aat agc caa gaa agt tca Asp Ser Lys Leu Leu Glu Ser Gly Leu Met Asn Ser Gln Glu Ser Ser 965 970 975			3105
tgg gga aaa aat gta tcg tca aca gag agt ggt agg tta ttt aaa ggg Trp Gly Lys Asn Val Ser Ser Thr Glu Ser Gly Arg Leu Phe Lys Gly 980 985 990			3153
aaa aga gct cat gga cct gct ttg ttg act aaa gat aat gcc tta Lys Arg Ala His Gly Pro Ala Leu Leu Thr Lys Asp Asn Ala Leu 995 1000 1005			3198
ttc aaa gtt agc atc tct ttg tta aag aca aac aaa act tcc aat Phe Lys Val Ser Ile Ser Leu Leu Lys Thr Asn Lys Thr Ser Asn 1010 1015 1020			3243
aat tca gca act aat aga aag act cac att gat ggc cca tca tta Asn Ser Ala Thr Asn Arg Lys Thr His Ile Asp Gly Pro Ser Leu 1025 1030 1035			3288
tta att gag aat agt cca tca gtc tgg caa aat ata tta gaa agt Leu Ile Glu Asn Ser Pro Ser Val Trp Gln Asn Ile Leu Glu Ser 1040 1045 1050			3333
gac act gag ttt aaa aaa gtg aca cct ttg att cat gac aga atg Asp Thr Glu Phe Lys Lys Val Thr Pro Leu Ile His Asp Arg Met 1055 1060 1065			3378
ctt atg gac aaa aat gct aca gct ttg agg cta aat cat atg tca Leu Met Asp Lys Asn Ala Thr Ala Leu Arg Leu Asn His Met Ser 1070 1075 1080			3423
aat aaa act act tca tca aaa aac atg gaa atg gtc caa cag aaa Asn Lys Thr Thr Ser Ser Lys Asn Met Glu Met Val Gln Gln Lys			3468

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1085	1090	1095	
aaa gag ggc ccc att cca cca gat gca caa aat cca gat atg tcg			3513
Lys Glu Gly Pro Ile Pro Pro Asp Ala Gln Asn Pro Asp Met Ser			
1100	1105	1110	
ttc ttt aag atg cta ttc ttg cca gaa tca gca agg tgg ata caa			3558
Phe Phe Lys Met Leu Phe Leu Pro Glu Ser Ala Arg Trp Ile Gln			
1115	1120	1125	
agg act cat gga aag aac tct ctg aac tct ggg caa ggc ccc agt			3603
Arg Thr His Gly Lys Asn Ser Leu Asn Ser Gly Gln Gly Pro Ser			
1130	1135	1140	
cca aag caa tta gta tcc tta gga cca gaa aaa tct gtg gaa ggt			3648
Pro Lys Gln Leu Val Ser Leu Gly Pro Glu Lys Ser Val Glu Gly			
1145	1150	1155	
cag aat ttc ttg tct gag aaa aac aaa gtg gta gta gga aag ggt			3693
Gln Asn Phe Leu Ser Glu Lys Asn Lys Val Val Val Gly Lys Gly			
1160	1165	1170	
gaa ttt aca aag gac gta gga ctc aaa gag atg gtt ttt cca agc			3738
Glu Phe Thr Lys Asp Val Gly Leu Lys Glu Met Val Phe Pro Ser			
1175	1180	1185	
agc aga aac cta ttt ctt act aac ttg gat aat tta cat gaa aat			3783
Ser Arg Asn Leu Phe Leu Thr Asn Leu Asp Asn Leu His Glu Asn			
1190	1195	1200	
aat aca cac aat caa gaa aaa aaa att cag gaa gaa ata gaa aag			3828
Asn Thr His Asn Gln Glu Lys Lys Ile Gln Glu Glu Ile Glu Lys			
1205	1210	1215	
aag gaa aca tta atc caa gag aat gta gtt ttg cct cag ata cat			3873
Lys Glu Thr Leu Ile Gln Glu Asn Val Val Leu Pro Gln Ile His			
1220	1225	1230	
aca gtg act ggc act aag aat ttc atg aag aac ctt ttc tta ctg			3918
Thr Val Thr Gly Thr Lys Asn Phe Met Lys Asn Leu Phe Leu Leu			
1235	1240	1245	
agc act agg caa aat gta gaa ggt tca tat gac ggg gca tat gct			3963
Ser Thr Arg Gln Asn Val Glu Gly Ser Tyr Asp Gly Ala Tyr Ala			
1250	1255	1260	
cca gta ctt caa gat ttt agg tca tta aat gat tca aca aat aga			4008
Pro Val Leu Gln Asp Phe Arg Ser Leu Asn Asp Ser Thr Asn Arg			
1265	1270	1275	
aca aag aaa cac aca gct cat ttc tca aaa aaa ggg gag gaa gaa			4053
Thr Lys Lys His Thr Ala His Phe Ser Lys Lys Gly Glu Glu Glu			
1280	1285	1290	
aac ttg gaa ggc ttg gga aat caa acc aag caa att gta gag aaa			4098
Asn Leu Glu Gly Leu Gly Asn Gln Thr Lys Gln Ile Val Glu Lys			
1295	1300	1305	
tat gca tgc acc aca agg ata tct cct aat aca agc cag cag aat			4143
Tyr Ala Cys Thr Thr Arg Ile Ser Pro Asn Thr Ser Gln Gln Asn			
1310	1315	1320	
ttt gtc acg caa cgt agt aag aga gct ttg aaa caa ttc aga ctc			4188
Phe Val Thr Gln Arg Ser Lys Arg Ala Leu Lys Gln Phe Arg Leu			
1325	1330	1335	
cca cta gaa gaa aca gaa ctt gaa aaa agg ata att gtg gat gac			4233
Pro Leu Glu Glu Thr Glu Leu Glu Lys Arg Ile Ile Val Asp Asp			
1340	1345	1350	
acc tca acc cag tgg tcc aaa aac atg aaa cat ttg acc ccg agc			4278
Thr Ser Thr Gln Trp Ser Lys Asn Met Lys His Leu Thr Pro Ser			
1355	1360	1365	
acc ctc aca cag ata gac tac aat gag aag gag aaa ggg gcc att			4323
Thr Leu Thr Gln Ile Asp Tyr Asn Glu Lys Glu Lys Gly Ala Ile			
1370	1375	1380	
act cag tct ccc tta tca gat tgc ctt acg agg agt cat agc atc			4368
Thr Gln Ser Pro Leu Ser Asp Cys Leu Thr Arg Ser His Ser Ile			

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1385		1390		1395		
cct	caa gca aat aga	tct	cca tta ccc att	gca	aag gta tca tca	4413
Pro	Gln Ala Asn Arg	Ser	Pro Leu Pro Ile	Ala	Lys Val Ser Ser	
1400		1405		1410		
ttt	cca tct att aga	cct	ata tat ctg acc	agg	gtc cta ttc caa	4458
Phe	Pro Ser Ile Arg	Pro	Ile Tyr Leu Thr	Arg	Val Leu Phe Gln	
1415		1420		1425		
gac	aac tct tct cat	ctt	cca gca gca tct	tat	aga aag aaa gat	4503
Asp	Asn Ser Ser His	Leu	Pro Ala Ala Ser	Tyr	Arg Lys Lys Asp	
1430		1435		1440		
tct	ggg gtc caa gaa	agc	agt cat ttc tta	caa	gga gcc aaa aaa	4548
Ser	Gly Val Gln Glu	Ser	Ser His Phe Leu	Gln	Gly Ala Lys Lys	
1445		1450		1455		
aat	aac ctt tct tta	gcc	att cta acc ttg	gag	atg act ggt gat	4593
Asn	Asn Leu Ser Leu	Ala	Ile Leu Thr Leu	Glu	Met Thr Gly Asp	
1460		1465		1470		
caa	aga gag gtt ggc	tcc	ctg ggg aca agt	gcc	aca aat tca gtc	4638
Gln	Arg Glu Val Gly	Ser	Leu Gly Thr Ser	Ala	Thr Asn Ser Val	
1475		1480		1485		
aca	tac aag aaa gtt	gag	aac act gtt ctc	ccg	aaa cca gac ttg	4683
Thr	Tyr Lys Lys Val	Glu	Asn Thr Val Leu	Pro	Lys Pro Asp Leu	
1490		1495		1500		
ccc	aaa aca tct ggc	aaa	gtt gaa ttg ctt	cca	aaa gtt cac att	4728
Pro	Lys Thr Ser Gly	Lys	Val Glu Leu Leu	Pro	Lys Val His Ile	
1505		1510		1515		
tat	cag aag gac cta	ttc	cct acg gaa act	agc	aat ggg tct cct	4773
Tyr	Gln Lys Asp Leu	Phe	Pro Thr Glu Thr	Ser	Asn Gly Ser Pro	
1520		1525		1530		
ggc	cat ctg gat ctc	gtg	gaa ggg agc ctt	ctt	cag gga aca gag	4818
Gly	His Leu Asp Leu	Val	Glu Gly Ser Leu	Leu	Gln Gly Thr Glu	
1535		1540		1545		
gga	gcg att aag tgg	aat	gaa gca aac aga	cct	gga aaa gtt ccc	4863
Gly	Ala Ile Lys Trp	Asn	Glu Ala Asn Arg	Pro	Gly Lys Val Pro	
1550		1555		1560		
ttt	ctg aga gta gca	aca	gaa agc tct gca	aag	act ccc tcc aag	4908
Phe	Leu Arg Val Ala	Thr	Glu Ser Ser Ala	Lys	Thr Pro Ser Lys	
1565		1570		1575		
cta	ttg gat cct ctt	gct	tgg gat aac cac	tat	ggt act cag ata	4953
Leu	Leu Asp Pro Leu	Ala	Trp Asp Asn His	Tyr	Gly Thr Gln Ile	
1580		1585		1590		
cca	aaa gaa gag tgg	aaa	tcc caa gag aag	tca	cca gaa aaa aca	4998
Pro	Lys Glu Glu Trp	Lys	Ser Gln Glu Lys	Ser	Pro Glu Lys Thr	
1595		1600		1605		
gct	ttt aag aaa aag	gat	acc att ttg tcc	ctg	aac gct tgt gaa	5043
Ala	Phe Lys Lys Lys	Asp	Thr Ile Leu Ser	Leu	Asn Ala Cys Glu	
1610		1615		1620		
agc	aat cat gca ata	gca	gca ata aat gag	gga	caa aat aag ccc	5088
Ser	Asn His Ala Ile	Ala	Ala Ile Asn Glu	Gly	Gln Asn Lys Pro	
1625		1630		1635		
gaa	ata gaa gtc acc	tgg	gca aag caa ggt	agg	act gaa agg ctg	5133
Glu	Ile Glu Val Thr	Trp	Ala Lys Gln Gly	Arg	Thr Glu Arg Leu	
1640		1645		1650		
tgc	tct caa aac cca	cca	gtc ttg aaa cgc	cat	caa cgg gaa ata	5178
Cys	Ser Gln Asn Pro	Pro	Val Leu Lys Arg	His	Gln Arg Glu Ile	
1655		1660		1665		
act	cgt act act ctt	cag	tca gat caa gag	gaa	att gac tat gat	5223
Thr	Arg Thr Thr Leu	Gln	Ser Asp Gln Glu	Glu	Ile Asp Tyr Asp	
1670		1675		1680		
gat	acc ata tca gtt	gaa	atg aag aag gaa	gat	ttt gac att tat	5268
Asp	Thr Ile Ser Val	Glu	Met Lys Lys Glu	Asp	Phe Asp Ile Tyr	

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1685	1690	1695	
gat gag gat gaa aat cag agc ccc cgc agc ttt caa aag aaa aca			5313
Asp Glu Asp Glu Asn Gln Ser Pro Arg Ser Phe Gln Lys Lys Thr			
1700	1705	1710	
cga cac tat ttt att gct gca gtg gag agg ctc tgg gat tat ggg			5358
Arg His Tyr Phe Ile Ala Val Glu Arg Leu Trp Asp Tyr Gly			
1715	1720	1725	
atg agt agc tcc cca cat gtt cta aga aac agg gct cag agt ggc			5403
Met Ser Ser Ser Pro His Val Leu Arg Asn Arg Ala Gln Ser Gly			
1730	1735	1740	
agt gtc cct cag ttc aag aaa gtt gtt ttc cag gaa ttt act gat			5448
Ser Val Pro Gln Phe Lys Lys Val Val Phe Gln Glu Phe Thr Asp			
1745	1750	1755	
ggc tcc ttt act cag ccc tta tac cgt gga gaa cta aat gaa cat			5493
Gly Ser Phe Thr Gln Pro Leu Tyr Arg Gly Glu Leu Asn Glu His			
1760	1765	1770	
ttg gga ctc ctg ggg cca tat ata aga gca gaa gtt gaa gat aat			5538
Leu Gly Leu Leu Gly Pro Tyr Ile Arg Ala Glu Val Glu Asp Asn			
1775	1780	1785	
atc atg gta act ttc aga aat cag gcc tct cgt ccc tat tcc ttc			5583
Ile Met Val Thr Phe Arg Asn Gln Ala Ser Arg Pro Tyr Ser Phe			
1790	1795	1800	
tat tct agc ctt att tct tat gag gaa gat cag agg caa gga gca			5628
Tyr Ser Ser Leu Ile Ser Tyr Glu Glu Asp Gln Arg Gln Gly Ala			
1805	1810	1815	
gaa cct aga aaa aac ttt gtc aag cct aat gaa acc aaa act tac			5673
Glu Pro Arg Lys Asn Phe Val Lys Pro Asn Glu Thr Lys Thr Tyr			
1820	1825	1830	
ttt tgg aaa gtg caa cat cat atg gca ccc act aaa gat gag ttt			5718
Phe Trp Lys Val Gln His His Met Ala Pro Thr Lys Asp Glu Phe			
1835	1840	1845	
gac tgc aaa gcc tgg gct tat ttc tct gat gtt gac ctg gaa aaa			5763
Asp Cys Lys Ala Trp Ala Tyr Phe Ser Asp Val Asp Leu Glu Lys			
1850	1855	1860	
gat gtg cac tca ggc ctg att gga ccc ctt ctg gtc tgc cac act			5808
Asp Val His Ser Gly Leu Ile Gly Pro Leu Leu Val Cys His Thr			
1865	1870	1875	
aac aca ctg aac cct gct cat ggg aga caa gtg aca gta cag gaa			5853
Asn Thr Leu Asn Pro Ala His Gly Arg Gln Val Thr Val Gln Glu			
1880	1885	1890	
ttt gct ctg ttt ttc acc atc ttt gat gag acc aaa agc tgg tac			5898
Phe Ala Leu Phe Phe Thr Ile Phe Asp Glu Thr Lys Ser Trp Tyr			
1895	1900	1905	
ttc act gaa aat atg gaa aga aac tgc agg gct ccc tgc aat atc			5943
Phe Thr Glu Asn Met Glu Arg Asn Cys Arg Ala Pro Cys Asn Ile			
1910	1915	1920	
cag atg gaa gat ccc act ttt aaa gag aat tat cgc ttc cat gca			5988
Gln Met Glu Asp Pro Thr Phe Lys Glu Asn Tyr Arg Phe His Ala			
1925	1930	1935	
atc aat ggc tac ata atg gat aca cta cct ggc tta gta atg gct			6033
Ile Asn Gly Tyr Ile Met Asp Thr Leu Pro Gly Leu Val Met Ala			
1940	1945	1950	
cag gat caa agg att cga tgg tat ctg ctc agc atg ggc agc aat			6078
Gln Asp Gln Arg Ile Arg Trp Tyr Leu Leu Ser Met Gly Ser Asn			
1955	1960	1965	
gaa aac atc cat tct att cat ttc agt gga cat gtg ttc act gta			6123
Glu Asn Ile His Ser Ile His Phe Ser Gly His Val Phe Thr Val			
1970	1975	1980	
cga aaa aaa gag gag tat aaa atg gca ctg tac aat ctc tat cca			6168
Arg Lys Lys Glu Glu Tyr Lys Met Ala Leu Tyr Asn Leu Tyr Pro			



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1985	1990	1995	
ggt gtt ttt gag aca Gly Val Phe Glu Thr 2000	gtg gaa atg tta cca tcc Val Glu Met Leu Pro Ser 2005	aaa gct gga att Lys Ala Gly Ile 2010	6213
tgg cgg gtg gaa tgc ctt Trp Arg Val Glu Cys 2015	att ggc gag cat cta Ile Gly Glu His Leu 2020	cat gct ggg atg His Ala Gly Met 2025	6258
agc aca ctt ttt ctg gtg Ser Thr Leu Phe Leu 2030	tac agc aat aag tgt Tyr Ser Asn Lys Cys 2035	cag act ccc ctg Gln Thr Pro Leu 2040	6303
gga atg gct tct gga cac Gly Met Ala Ser Gly 2045	att aga gat ttt cag Ile Arg Asp Phe Gln 2050	att aca gct tca Ile Thr Ala Ser 2055	6348
gga caa tat gga cag tgg Gly Gln Tyr Gly Gln 2060	gcc cca aag ctg gcc Ala Pro Lys Leu Ala 2065	aga ctt cat tat Arg Leu His Tyr 2070	6393
tcc gga tca atc aat gcc Ser Gly Ser Ile Asn 2075	tgg agc acc aag gag Trp Ser Thr Lys Glu 2080	ccc ttt tct tgg Pro Phe Ser Trp 2085	6438
atc aag gtg gat ctg ttg Ile Lys Val Asp Leu 2090	gca cca atg att att Ala Pro Met Ile Ile 2095	cac ggc atc aag His Gly Ile Lys 2100	6483
acc cag ggt gcc cgt cag Thr Gln Gly Ala Arg 2105	aag ttc tcc agc ctc Lys Phe Ser Ser Leu 2110	tac atc tct cag Tyr Ile Ser Gln 2115	6528
ttt atc atc atg tat agt Phe Ile Ile Met Tyr 2120	ctt gat ggg aag aag Leu Asp Gly Lys Lys 2125	tgg cag act tat Trp Gln Thr Tyr 2130	6573
cga gga aat tcc act gga Arg Gly Asn Ser Thr 2135	acc tta atg gtc ttc Thr Leu Met Val Phe 2140	ttt ggc aat gtg Phe Gly Asn Val 2145	6618
gat tca tct ggg ata aaa Asp Ser Ser Gly Ile 2150	cac aat att ttt aac His Asn Ile Phe Asn 2155	cct cca att att Pro Pro Ile Ile 2160	6663
gct cga tac atc cgt ttg Ala Arg Tyr Ile Arg 2165	cac cca act cat tat His Pro Thr His Tyr 2170	agc att cgc agc Ser Ile Arg Ser 2175	6708
act ctt cgc atg gag ttg Thr Leu Arg Met Glu 2180	atg ggc tgt gat tta Met Gly Cys Asp Leu 2185	aat agt tgc agc Asn Ser Cys Ser 2190	6753
atg cca ttg gga atg gag Met Pro Leu Gly Met 2195	agt aaa gca ata tca Ser Lys Ala Ile Ser 2200	gat gca cag att Asp Ala Gln Ile 2205	6798
act gct tca tcc tac ttt Thr Ala Ser Ser Tyr 2210	acc aat atg ttt gcc Thr Asn Met Phe Ala 2215	acc tgg tct cct Thr Trp Ser Pro 2220	6843
tca aaa gct cga ctt cac Ser Lys Ala Arg Leu 2225	ctc caa ggg agg agt Leu Gln Gly Arg Ser 2230	aat gcc tgg aga Asn Ala Trp Arg 2235	6888
cct cag gtg aat aat cca Pro Gln Val Asn Asn 2240	aaa gag tgg ctg caa Lys Glu Trp Leu Gln 2245	gtg gac ttc cag Val Asp Phe Gln 2250	6933
aag aca atg aaa gtc aca Lys Thr Met Lys Val 2255	gga gta act act cag Gly Val Thr Thr Gln 2260	gga gta aaa tct Gly Val Lys Ser 2265	6978
ctg ctt acc agc atg tat Leu Leu Thr Ser Met 2270	gtg aag gag ttc ctc Val Lys Glu Phe Leu 2275	atc tcc agc agt Ile Ser Ser Ser 2280	7023
caa gat ggc cat cag tgg Gln Asp Gly His Gln 2285	act ctc ttt ttt cag Thr Leu Phe Phe Gln 2290	aat ggc aaa gta Asn Gly Lys Val 2295	7068

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Lys Val Phe Gln Gly Asn Gln Asp Ser Phe Thr Pro Val Val Asn			
2300	2305	2310	
tct cta gac cca ccg tta ctg act cgc tac ctt cga att cac ccc			7158
Ser Leu Asp Pro Pro Leu Leu Thr Arg Tyr Leu Arg Ile His Pro			
2315	2320	2325	
cag agt tgg gtg cac cag att gcc ctg agg atg gag gtt ctg ggc			7203
Gln Ser Trp Val His Gln Ile Ala Leu Arg Met Glu Val Leu Gly			
2330	2335	2340	
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Cys Glu Ala Gln Asp Leu Tyr			
2345	2350		
gccactgccg tcacctctcc ctectcagct ccagggcagt gtcctcctt ggcttgcctt			7307
ctacctttgt gctaaatcct agcagacact gccttgaagc ctctgaatt aactatcatc			7367
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gcactcagtt tactctctcc ctctactaat ttctgctga aaataacaca acaaaaatgt			8267
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caaggaggtc agaagaaaat tggactggtg aaaacagaaa aaacactcca gtctgccata			8387
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ccagggcaaa tggaaaacag gagatcctaa tatgaaagaa aaatggatcc caatctgaga			8627
aaaggcaaaa gaatggctac ttttttctat gctggagtat tttctaataa tctgcttga			8687
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<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

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          20          25          30
Trp  Asp  Tyr  Met  Gln  Ser  Asp  Leu  Gly  Glu  Leu  Pro  Val  Asp  Ala  Arg
          35          40          45
Phe  Pro  Pro  Arg  Val  Pro  Lys  Ser  Phe  Pro  Phe  Asn  Thr  Ser  Val  Val
          50          55          60
Tyr  Lys  Lys  Thr  Leu  Phe  Val  Glu  Phe  Thr  Asp  His  Leu  Phe  Asn  Ile
65          70          75          80
Ala  Lys  Pro  Arg  Pro  Pro  Trp  Met  Gly  Leu  Leu  Gly  Pro  Thr  Ile  Gln
          85          90          95
Ala  Glu  Val  Tyr  Asp  Thr  Val  Val  Ile  Thr  Leu  Lys  Asn  Met  Ala  Ser
          100         105         110
His  Pro  Val  Ser  Leu  His  Ala  Val  Gly  Val  Ser  Tyr  Trp  Lys  Ala  Ser
          115         120         125
Glu  Gly  Ala  Glu  Tyr  Asp  Asp  Gln  Thr  Ser  Gln  Arg  Glu  Lys  Glu  Asp
          130         135         140
Asp  Lys  Val  Phe  Pro  Gly  Gly  Ser  His  Thr  Tyr  Val  Trp  Gln  Val  Leu
145         150         155         160
Lys  Glu  Asn  Gly  Pro  Met  Ala  Ser  Asp  Pro  Leu  Cys  Leu  Thr  Tyr  Ser
          165         170         175
Tyr  Leu  Ser  His  Val  Asp  Leu  Val  Lys  Asp  Leu  Asn  Ser  Gly  Leu  Ile
          180         185         190
Gly  Ala  Leu  Leu  Val  Cys  Arg  Glu  Gly  Ser  Leu  Ala  Lys  Glu  Lys  Thr
          195         200         205
Gln  Thr  Leu  His  Lys  Phe  Ile  Leu  Leu  Phe  Ala  Val  Phe  Asp  Glu  Gly
          210         215         220
Lys  Ser  Trp  His  Ser  Glu  Thr  Lys  Asn  Ser  Leu  Met  Gln  Asp  Arg  Asp
225         230         235         240
Ala  Ala  Ser  Ala  Arg  Ala  Trp  Pro  Lys  Met  His  Thr  Val  Asn  Gly  Tyr
          245         250         255
Val  Asn  Arg  Ser  Leu  Pro  Gly  Leu  Ile  Gly  Cys  His  Arg  Lys  Ser  Val
          260         265         270
Tyr  Trp  His  Val  Ile  Gly  Met  Gly  Thr  Thr  Pro  Glu  Val  His  Ser  Ile
          275         280         285
Phe  Leu  Glu  Gly  His  Thr  Phe  Leu  Val  Arg  Asn  His  Arg  Gln  Ala  Ser
          290         295         300
Leu  Glu  Ile  Ser  Pro  Ile  Thr  Phe  Leu  Thr  Ala  Gln  Thr  Leu  Leu  Met
305         310         315         320
Asp  Leu  Gly  Gln  Phe  Leu  Leu  Phe  Cys  His  Ile  Ser  Ser  His  Gln  His
          325         330         335
Asp  Gly  Met  Glu  Ala  Tyr  Val  Lys  Val  Asp  Ser  Cys  Pro  Glu  Glu  Pro
          340         345         350
Gln  Leu  Arg  Met  Lys  Asn  Asn  Glu  Glu  Ala  Glu  Asp  Tyr  Asp  Asp  Asp
          355         360         365
Leu  Thr  Asp  Ser  Glu  Met  Asp  Val  Val  Arg  Phe  Asp  Asp  Asp  Asn  Ser
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Pro  Ser  Phe  Ile  Gln  Ile  Arg  Ser  Val  Ala  Lys  Lys  His  Pro  Lys  Thr
385         390         395         400

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Trp Val His Tyr Ile Ala Ala Glu Glu Glu Asp Trp Asp Tyr Ala Pro  
 405 410 415  
 Leu Val Leu Ala Pro Asp Asp Arg Ser Tyr Lys Ser Gln Tyr Leu Asn  
 420 425 430  
 Asn Gly Pro Gln Arg Ile Gly Arg Lys Tyr Lys Lys Val Arg Phe Met  
 435 440 445  
 Ala Tyr Thr Asp Glu Thr Phe Lys Thr Arg Glu Ala Ile Gln His Glu  
 450 455 460  
 Ser Gly Ile Leu Gly Pro Leu Leu Tyr Gly Glu Val Gly Asp Thr Leu  
 465 470 475 480  
 Leu Ile Ile Phe Lys Asn Gln Ala Ser Arg Pro Tyr Asn Ile Tyr Pro  
 485 490 495  
 His Gly Ile Thr Asp Val Arg Pro Leu Tyr Ser Arg Arg Leu Pro Lys  
 500 505 510  
 Gly Val Lys His Leu Lys Asp Phe Pro Ile Leu Pro Gly Glu Ile Phe  
 515 520 525  
 Lys Tyr Lys Trp Thr Val Thr Val Glu Asp Gly Pro Thr Lys Ser Asp  
 530 535 540  
 Pro Arg Cys Leu Thr Arg Tyr Tyr Ser Ser Phe Val Asn Met Glu Arg  
 545 550 555 560  
 Asp Leu Ala Ser Gly Leu Ile Gly Pro Leu Leu Ile Cys Tyr Lys Glu  
 565 570 575  
 Ser Val Asp Gln Arg Gly Asn Gln Ile Met Ser Asp Lys Arg Asn Val  
 580 585 590  
 Ile Leu Phe Ser Val Phe Asp Glu Asn Arg Ser Trp Tyr Leu Thr Glu  
 595 600 605  
 Asn Ile Gln Arg Phe Leu Pro Asn Pro Ala Gly Val Gln Leu Glu Asp  
 610 615 620  
 Pro Glu Phe Gln Ala Ser Asn Ile Met His Ser Ile Asn Gly Tyr Val  
 625 630 635 640  
 Phe Asp Ser Leu Gln Leu Ser Val Cys Leu His Glu Val Ala Tyr Trp  
 645 650 655  
 Tyr Ile Leu Ser Ile Gly Ala Gln Thr Asp Phe Leu Ser Val Phe Phe  
 660 665 670  
 Ser Gly Tyr Thr Phe Lys His Lys Met Val Tyr Glu Asp Thr Leu Thr  
 675 680 685  
 Leu Phe Pro Phe Ser Gly Glu Thr Val Phe Met Ser Met Glu Asn Pro  
 690 695 700  
 Gly Leu Trp Ile Leu Gly Cys His Asn Ser Asp Phe Arg Asn Arg Gly  
 705 710 715 720  
 Met Thr Ala Leu Leu Lys Val Ser Ser Cys Asp Lys Asn Thr Gly Asp  
 725 730 735  
 Tyr Tyr Glu Asp Ser Tyr Glu Asp Ile Ser Ala Tyr Leu Leu Ser Lys  
 740 745 750  
 Asn Asn Ala Ile Glu Pro Arg Ser Phe Ser Gln Asn Ser Arg His Pro  
 755 760 765  
 Ser Thr Arg Gln Lys Gln Phe Asn Ala Thr Thr Ile Pro Glu Asn Asp  
 770 775 780  
 Ile Glu Lys Thr Asp Pro Trp Phe Ala His Arg Thr Pro Met Pro Lys  
 785 790 795 800  
 Ile Gln Asn Val Ser Ser Ser Asp Leu Leu Met Leu Leu Arg Gln Ser  
 805 810 815  
 Pro Thr Pro His Gly Leu Ser Leu Ser Asp Leu Gln Glu Ala Lys Tyr

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820				825				830							
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Ser	Leu	Ser	Glu	Met	Thr	His	Phe	Arg	Pro	Gln	Leu	His	His	Ser	Gly
	850					855					860				
Asp	Met	Val	Phe	Thr	Pro	Glu	Ser	Gly	Leu	Gln	Leu	Arg	Leu	Asn	Glu
865					870					875					880
Lys	Leu	Gly	Thr	Thr	Ala	Ala	Thr	Glu	Leu	Lys	Lys	Leu	Asp	Phe	Lys
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Val	Ser	Ser	Thr	Ser	Asn	Asn	Leu	Ile	Ser	Thr	Ile	Pro	Ser	Asp	Asn
			900					905					910		
Leu	Ala	Ala	Gly	Thr	Asp	Asn	Thr	Ser	Ser	Leu	Gly	Pro	Pro	Ser	Met
		915					920					925			
Pro	Val	His	Tyr	Asp	Ser	Gln	Leu	Asp	Thr	Thr	Leu	Phe	Gly	Lys	Lys
	930					935					940				
Ser	Ser	Pro	Leu	Thr	Glu	Ser	Gly	Gly	Pro	Leu	Ser	Leu	Ser	Glu	Glu
945					950					955					960
Asn	Asn	Asp	Ser	Lys	Leu	Leu	Glu	Ser	Gly	Leu	Met	Asn	Ser	Gln	Glu
				965					970					975	
Ser	Ser	Trp	Gly	Lys	Asn	Val	Ser	Ser	Thr	Glu	Ser	Gly	Arg	Leu	Phe
			980					985					990		
Lys	Gly	Lys	Arg	Ala	His	Gly	Pro	Ala	Leu	Leu	Thr	Lys	Asp	Asn	Ala
		995					1000						1005		
Leu	Phe	Lys	Val	Ser	Ile	Ser	Leu	Leu	Lys	Thr	Asn	Lys	Thr	Ser	
	1010					1015					1020				
Asn	Asn	Ser	Ala	Thr	Asn	Arg	Lys	Thr	His	Ile	Asp	Gly	Pro	Ser	
	1025					1030					1035				
Leu	Leu	Ile	Glu	Asn	Ser	Pro	Ser	Val	Trp	Gln	Asn	Ile	Leu	Glu	
	1040					1045					1050				
Ser	Asp	Thr	Glu	Phe	Lys	Lys	Val	Thr	Pro	Leu	Ile	His	Asp	Arg	
	1055					1060					1065				
Met	Leu	Met	Asp	Lys	Asn	Ala	Thr	Ala	Leu	Arg	Leu	Asn	His	Met	
	1070					1075					1080				
Ser	Asn	Lys	Thr	Thr	Ser	Ser	Lys	Asn	Met	Glu	Met	Val	Gln	Gln	
	1085					1090					1095				
Lys	Lys	Glu	Gly	Pro	Ile	Pro	Pro	Asp	Ala	Gln	Asn	Pro	Asp	Met	
	1100					1105					1110				
Ser	Phe	Phe	Lys	Met	Leu	Phe	Leu	Pro	Glu	Ser	Ala	Arg	Trp	Ile	
	1115					1120					1125				
Gln	Arg	Thr	His	Gly	Lys	Asn	Ser	Leu	Asn	Ser	Gly	Gln	Gly	Pro	
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Ser	Pro	Lys	Gln	Leu	Val	Ser	Leu	Gly	Pro	Glu	Lys	Ser	Val	Glu	
	1145					1150					1155				
Gly	Gln	Asn	Phe	Leu	Ser	Glu	Lys	Asn	Lys	Val	Val	Val	Gly	Lys	
	1160					1165					1170				
Gly	Glu	Phe	Thr	Lys	Asp	Val	Gly	Leu	Lys	Glu	Met	Val	Phe	Pro	
	1175					1180					1185				
Ser	Ser	Arg	Asn	Leu	Phe	Leu	Thr	Asn	Leu	Asp	Asn	Leu	His	Glu	
	1190					1195					1200				
Asn	Asn	Thr	His	Asn	Gln	Glu	Lys	Lys	Ile	Gln	Glu	Glu	Ile	Glu	
	1205					1210					1215				
Lys	Lys	Glu	Thr	Leu	Ile	Gln	Glu	Asn	Val	Val	Leu	Pro	Gln	Ile	
	1220					1225					1230				

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His Thr	Val Thr Gly Thr	Lys	Asn Phe Met Lys	Asn	Leu Phe Leu
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Leu Ser	Thr Arg Gln Asn Val	Glu Gly Ser Tyr	Asp	Gly Ala Tyr	
1250		1255	1260		
Ala Pro	Val Leu Gln Asp Phe	Arg Ser Leu Asn	Asp	Ser Thr Asn	
1265		1270	1275		
Arg Thr	Lys Lys His Thr Ala	His Phe Ser Lys	Lys	Gly Glu Glu	
1280		1285	1290		
Glu Asn	Leu Glu Gly Leu Gly	Asn Gln Thr Lys	Gln	Ile Val Glu	
1295		1300	1305		
Lys Tyr	Ala Cys Thr Thr Arg	Ile Ser Pro Asn	Thr	Ser Gln Gln	
1310		1315	1320		
Asn Phe	Val Thr Gln Arg Ser	Lys Arg Ala Leu	Lys	Gln Phe Arg	
1325		1330	1335		
Leu Pro	Leu Glu Glu Thr Glu	Leu Glu Lys Arg	Ile	Ile Val Asp	
1340		1345	1350		
Asp Thr	Ser Thr Gln Trp Ser	Lys Asn Met Lys	His	Leu Thr Pro	
1355		1360	1365		
Ser Thr	Leu Thr Gln Ile Asp	Tyr Asn Glu Lys	Glu	Lys Gly Ala	
1370		1375	1380		
Ile Thr	Gln Ser Pro Leu Ser	Asp Cys Leu Thr	Arg	Ser His Ser	
1385		1390	1395		
Ile Pro	Gln Ala Asn Arg Ser	Pro Leu Pro Ile	Ala	Lys Val Ser	
1400		1405	1410		
Ser Phe	Pro Ser Ile Arg Pro	Ile Tyr Leu Thr	Arg	Val Leu Phe	
1415		1420	1425		
Gln Asp	Asn Ser Ser His Leu	Pro Ala Ala Ser	Tyr	Arg Lys Lys	
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Asp Ser	Gly Val Gln Glu Ser	Ser His Phe Leu	Gln	Gly Ala Lys	
1445		1450	1455		
Lys Asn	Asn Leu Ser Leu Ala	Ile Leu Thr Leu	Glu	Met Thr Gly	
1460		1465	1470		
Asp Gln	Arg Glu Val Gly Ser	Leu Gly Thr Ser	Ala	Thr Asn Ser	
1475		1480	1485		
Val Thr	Tyr Lys Lys Val Glu	Asn Thr Val Leu	Pro	Lys Pro Asp	
1490		1495	1500		
Leu Pro	Lys Thr Ser Gly Lys	Val Glu Leu Leu	Pro	Lys Val His	
1505		1510	1515		
Ile Tyr	Gln Lys Asp Leu Phe	Pro Thr Glu Thr	Ser	Asn Gly Ser	
1520		1525	1530		
Pro Gly	His Leu Asp Leu Val	Glu Gly Ser Leu	Leu	Gln Gly Thr	
1535		1540	1545		
Glu Gly	Ala Ile Lys Trp Asn	Glu Ala Asn Arg	Pro	Gly Lys Val	
1550		1555	1560		
Pro Phe	Leu Arg Val Ala Thr	Glu Ser Ser Ala	Lys	Thr Pro Ser	
1565		1570	1575		
Lys Leu	Leu Asp Pro Leu Ala	Trp Asp Asn His	Tyr	Gly Thr Gln	
1580		1585	1590		
Ile Pro	Lys Glu Glu Trp Lys	Ser Gln Glu Lys	Ser	Pro Glu Lys	
1595		1600	1605		
Thr Ala	Phe Lys Lys Lys Asp	Thr Ile Leu Ser	Leu	Asn Ala Cys	
1610		1615	1620		
Glu Ser	Asn His Ala Ile Ala	Ala Ile Asn Glu	Gly	Gln Asn Lys	
1625		1630	1635		

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Pro	Glu	Ile	Glu	Val	Thr	Trp	Ala	Lys	Gln	Gly	Arg	Thr	Glu	Arg
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1655						1660					1665			
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1670						1675					1680			
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Tyr	Asp	Glu	Asp	Glu	Asn	Gln	Ser	Pro	Arg	Ser	Phe	Gln	Lys	Lys
1700						1705					1710			
Thr	Arg	His	Tyr	Phe	Ile	Ala	Ala	Val	Glu	Arg	Leu	Trp	Asp	Tyr
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Asp	Gly	Ser	Phe	Thr	Gln	Pro	Leu	Tyr	Arg	Gly	Glu	Leu	Asn	Glu
1760						1765					1770			
His	Leu	Gly	Leu	Leu	Gly	Pro	Tyr	Ile	Arg	Ala	Glu	Val	Glu	Asp
1775						1780					1785			
Asn	Ile	Met	Val	Thr	Phe	Arg	Asn	Gln	Ala	Ser	Arg	Pro	Tyr	Ser
1790						1795					1800			
Phe	Tyr	Ser	Ser	Leu	Ile	Ser	Tyr	Glu	Glu	Asp	Gln	Arg	Gln	Gly
1805						1810					1815			
Ala	Glu	Pro	Arg	Lys	Asn	Phe	Val	Lys	Pro	Asn	Glu	Thr	Lys	Thr
1820						1825					1830			
Tyr	Phe	Trp	Lys	Val	Gln	His	His	Met	Ala	Pro	Thr	Lys	Asp	Glu
1835						1840					1845			
Phe	Asp	Cys	Lys	Ala	Trp	Ala	Tyr	Phe	Ser	Asp	Val	Asp	Leu	Glu
1850						1855					1860			
Lys	Asp	Val	His	Ser	Gly	Leu	Ile	Gly	Pro	Leu	Leu	Val	Cys	His
1865						1870					1875			
Thr	Asn	Thr	Leu	Asn	Pro	Ala	His	Gly	Arg	Gln	Val	Thr	Val	Gln
1880						1885					1890			
Glu	Phe	Ala	Leu	Phe	Phe	Thr	Ile	Phe	Asp	Glu	Thr	Lys	Ser	Trp
1895						1900					1905			
Tyr	Phe	Thr	Glu	Asn	Met	Glu	Arg	Asn	Cys	Arg	Ala	Pro	Cys	Asn
1910						1915					1920			
Ile	Gln	Met	Glu	Asp	Pro	Thr	Phe	Lys	Glu	Asn	Tyr	Arg	Phe	His
1925						1930					1935			
Ala	Ile	Asn	Gly	Tyr	Ile	Met	Asp	Thr	Leu	Pro	Gly	Leu	Val	Met
1940						1945					1950			
Ala	Gln	Asp	Gln	Arg	Ile	Arg	Trp	Tyr	Leu	Leu	Ser	Met	Gly	Ser
1955						1960					1965			
Asn	Glu	Asn	Ile	His	Ser	Ile	His	Phe	Ser	Gly	His	Val	Phe	Thr
1970						1975					1980			
Val	Arg	Lys	Lys	Glu	Glu	Tyr	Lys	Met	Ala	Leu	Tyr	Asn	Leu	Tyr
1985						1990					1995			
Pro	Gly	Val	Phe	Glu	Thr	Val	Glu	Met	Leu	Pro	Ser	Lys	Ala	Gly
2000						2005					2010			
Ile	Trp	Arg	Val	Glu	Cys	Leu	Ile	Gly	Glu	His	Leu	His	Ala	Gly
2015						2020					2025			
Met	Ser	Thr	Leu	Phe	Leu	Val	Tyr	Ser	Asn	Lys	Cys	Gln	Thr	Pro

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2030	2035	2040
Leu Gly Met Ala Ser Gly His Ile Arg Asp Phe Gln Ile Thr Ala 2045	2050	2055
Ser Gly Gln Tyr Gly Gln Trp Ala Pro Lys Leu Ala Arg Leu His 2060	2065	2070
Tyr Ser Gly Ser Ile Asn Ala Trp Ser Thr Lys Glu Pro Phe Ser 2075	2080	2085
Trp Ile Lys Val Asp Leu Leu Ala Pro Met Ile Ile His Gly Ile 2090	2095	2100
Lys Thr Gln Gly Ala Arg Gln Lys Phe Ser Ser Leu Tyr Ile Ser 2105	2110	2115
Gln Phe Ile Ile Met Tyr Ser Leu Asp Gly Lys Lys Trp Gln Thr 2120	2125	2130
Tyr Arg Gly Asn Ser Thr Gly Thr Leu Met Val Phe Phe Gly Asn 2135	2140	2145
Val Asp Ser Ser Gly Ile Lys His Asn Ile Phe Asn Pro Pro Ile 2150	2155	2160
Ile Ala Arg Tyr Ile Arg Leu His Pro Thr His Tyr Ser Ile Arg 2165	2170	2175
Ser Thr Leu Arg Met Glu Leu Met Gly Cys Asp Leu Asn Ser Cys 2180	2185	2190
Ser Met Pro Leu Gly Met Glu Ser Lys Ala Ile Ser Asp Ala Gln 2195	2200	2205
Ile Thr Ala Ser Ser Tyr Phe Thr Asn Met Phe Ala Thr Trp Ser 2210	2215	2220
Pro Ser Lys Ala Arg Leu His Leu Gln Gly Arg Ser Asn Ala Trp 2225	2230	2235
Arg Pro Gln Val Asn Asn Pro Lys Glu Trp Leu Gln Val Asp Phe 2240	2245	2250
Gln Lys Thr Met Lys Val Thr Gly Val Thr Thr Gln Gly Val Lys 2255	2260	2265
Ser Leu Leu Thr Ser Met Tyr Val Lys Glu Phe Leu Ile Ser Ser 2270	2275	2280
Ser Gln Asp Gly His Gln Trp Thr Leu Phe Phe Gln Asn Gly Lys 2285	2290	2295
Val Lys Val Phe Gln Gly Asn Gln Asp Ser Phe Thr Pro Val Val 2300	2305	2310
Asn Ser Leu Asp Pro Pro Leu Leu Thr Arg Tyr Leu Arg Ile His 2315	2320	2325
Pro Gln Ser Trp Val His Gln Ile Ala Leu Arg Met Glu Val Leu 2330	2335	2340
Gly Cys Glu Ala Gln Asp Leu Tyr 2345	2350	

&lt;210&gt; SEQ ID NO 3

&lt;211&gt; LENGTH: 2332

&lt;212&gt; TYPE: PRT

&lt;213&gt; ORGANISM: Homo sapiens

&lt;400&gt; SEQUENCE: 3

Ala Thr Arg Arg Tyr Tyr Leu Gly Ala Val Glu Leu Ser Trp Asp Tyr  
1 5 10 15

Met Gln Ser Asp Leu Gly Glu Leu Pro Val Asp Ala Arg Phe Pro Pro  
20 25 30

Arg Val Pro Lys Ser Phe Pro Phe Asn Thr Ser Val Val Tyr Lys Lys



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35				40				45							
Thr	Leu	Phe	Val	Glu	Phe	Thr	Asp	His	Leu	Phe	Asn	Ile	Ala	Lys	Pro
50						55					60				
Arg	Pro	Pro	Trp	Met	Gly	Leu	Leu	Gly	Pro	Thr	Ile	Gln	Ala	Glu	Val
65				70					75					80	
Tyr	Asp	Thr	Val	Val	Ile	Thr	Leu	Lys	Asn	Met	Ala	Ser	His	Pro	Val
				85					90					95	
Ser	Leu	His	Ala	Val	Gly	Val	Ser	Tyr	Trp	Lys	Ala	Ser	Glu	Gly	Ala
			100					105					110		
Glu	Tyr	Asp	Asp	Gln	Thr	Ser	Gln	Arg	Glu	Lys	Glu	Asp	Asp	Lys	Val
		115					120					125			
Phe	Pro	Gly	Gly	Ser	His	Thr	Tyr	Val	Trp	Gln	Val	Leu	Lys	Glu	Asn
130						135					140				
Gly	Pro	Met	Ala	Ser	Asp	Pro	Leu	Cys	Leu	Thr	Tyr	Ser	Tyr	Leu	Ser
145					150					155					160
His	Val	Asp	Leu	Val	Lys	Asp	Leu	Asn	Ser	Gly	Leu	Ile	Gly	Ala	Leu
				165					170					175	
Leu	Val	Cys	Arg	Glu	Gly	Ser	Leu	Ala	Lys	Glu	Lys	Thr	Gln	Thr	Leu
			180					185					190		
His	Lys	Phe	Ile	Leu	Leu	Phe	Ala	Val	Phe	Asp	Glu	Gly	Lys	Ser	Trp
		195					200					205			
His	Ser	Glu	Thr	Lys	Asn	Ser	Leu	Met	Gln	Asp	Arg	Asp	Ala	Ala	Ser
		210				215					220				
Ala	Arg	Ala	Trp	Pro	Lys	Met	His	Thr	Val	Asn	Gly	Tyr	Val	Asn	Arg
225					230					235					240
Ser	Leu	Pro	Gly	Leu	Ile	Gly	Cys	His	Arg	Lys	Ser	Val	Tyr	Trp	His
				245					250					255	
Val	Ile	Gly	Met	Gly	Thr	Thr	Pro	Glu	Val	His	Ser	Ile	Phe	Leu	Glu
			260					265					270		
Gly	His	Thr	Phe	Leu	Val	Arg	Asn	His	Arg	Gln	Ala	Ser	Leu	Glu	Ile
		275					280					285			
Ser	Pro	Ile	Thr	Phe	Leu	Thr	Ala	Gln	Thr	Leu	Leu	Met	Asp	Leu	Gly
		290				295					300				
Gln	Phe	Leu	Leu	Phe	Cys	His	Ile	Ser	Ser	His	Gln	His	Asp	Gly	Met
305					310					315					320
Glu	Ala	Tyr	Val	Lys	Val	Asp	Ser	Cys	Pro	Glu	Glu	Pro	Gln	Leu	Arg
				325					330					335	
Met	Lys	Asn	Asn	Glu	Glu	Ala	Glu	Asp	Tyr	Asp	Asp	Asp	Leu	Thr	Asp
			340					345					350		
Ser	Glu	Met	Asp	Val	Val	Arg	Phe	Asp	Asp	Asp	Asp	Asn	Ser	Pro	Ser
		355					360					365			
Ile	Gln	Ile	Arg	Ser	Val	Ala	Lys	Lys	His	Pro	Lys	Thr	Trp	Val	His
		370				375					380				
Tyr	Ile	Ala	Ala	Glu	Glu	Glu	Asp	Trp	Asp	Tyr	Ala	Pro	Leu	Val	Leu
385					390				395						400
Ala	Pro	Asp	Asp	Arg	Ser	Tyr	Lys	Ser	Gln	Tyr	Leu	Asn	Asn	Gly	Pro
				405					410					415	
Gln	Arg	Ile	Gly	Arg	Lys	Tyr	Lys	Lys	Val	Arg	Phe	Met	Ala	Tyr	Thr
			420					425					430		
Asp	Glu	Thr	Phe	Lys	Thr	Arg	Glu	Ala	Ile	Gln	His	Glu	Ser	Gly	Ile
		435					440					445			
Leu	Gly	Pro	Leu	Leu	Tyr	Gly	Glu	Val	Gly	Asp	Thr	Leu	Leu	Ile	Ile
						455					460				

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Phe	Lys	Asn	Gln	Ala	Ser	Arg	Pro	Tyr	Asn	Ile	Tyr	Pro	His	Gly	Ile
465					470				475						480
Thr	Asp	Val	Arg	Pro	Leu	Tyr	Ser	Arg	Arg	Leu	Pro	Lys	Gly	Val	Lys
				485					490					495	
His	Leu	Lys	Asp	Phe	Pro	Ile	Leu	Pro	Gly	Glu	Ile	Phe	Lys	Tyr	Lys
			500					505					510		
Trp	Thr	Val	Thr	Val	Glu	Asp	Gly	Pro	Thr	Lys	Ser	Asp	Pro	Arg	Cys
		515					520					525			
Leu	Thr	Arg	Tyr	Tyr	Ser	Ser	Phe	Val	Asn	Met	Glu	Arg	Asp	Leu	Ala
	530					535					540				
Ser	Gly	Leu	Ile	Gly	Pro	Leu	Leu	Ile	Cys	Tyr	Lys	Glu	Ser	Val	Asp
545					550				555						560
Gln	Arg	Gly	Asn	Gln	Ile	Met	Ser	Asp	Lys	Arg	Asn	Val	Ile	Leu	Phe
				565					570					575	
Ser	Val	Phe	Asp	Glu	Asn	Arg	Ser	Trp	Tyr	Leu	Thr	Glu	Asn	Ile	Gln
			580					585						590	
Arg	Phe	Leu	Pro	Asn	Pro	Ala	Gly	Val	Gln	Leu	Glu	Asp	Pro	Glu	Phe
		595					600					605			
Gln	Ala	Ser	Asn	Ile	Met	His	Ser	Ile	Asn	Gly	Tyr	Val	Phe	Asp	Ser
	610					615					620				
Leu	Gln	Leu	Ser	Val	Cys	Leu	His	Glu	Val	Ala	Tyr	Trp	Tyr	Ile	Leu
625					630					635					640
Ser	Ile	Gly	Ala	Gln	Thr	Asp	Phe	Leu	Ser	Val	Phe	Phe	Ser	Gly	Tyr
				645					650					655	
Thr	Phe	Lys	His	Lys	Met	Val	Tyr	Glu	Asp	Thr	Leu	Thr	Leu	Phe	Pro
			660					665						670	
Phe	Ser	Gly	Glu	Thr	Val	Phe	Met	Ser	Met	Glu	Asn	Pro	Gly	Leu	Trp
		675					680					685			
Ile	Leu	Gly	Cys	His	Asn	Ser	Asp	Phe	Arg	Asn	Arg	Gly	Met	Thr	Ala
	690					695					700				
Leu	Leu	Lys	Val	Ser	Ser	Cys	Asp	Lys	Asn	Thr	Gly	Asp	Tyr	Tyr	Glu
705					710					715					720
Asp	Ser	Tyr	Glu	Asp	Ile	Ser	Ala	Tyr	Leu	Leu	Ser	Lys	Asn	Asn	Ala
				725					730					735	
Ile	Glu	Pro	Arg	Ser	Phe	Ser	Gln	Asn	Ser	Arg	His	Pro	Ser	Thr	Arg
			740					745						750	
Gln	Lys	Gln	Phe	Asn	Ala	Thr	Thr	Ile	Pro	Glu	Asn	Asp	Ile	Glu	Lys
		755						760				765			
Thr	Asp	Pro	Trp	Phe	Ala	His	Arg	Thr	Pro	Met	Pro	Lys	Ile	Gln	Asn
	770					775					780				
Val	Ser	Ser	Ser	Asp	Leu	Leu	Met	Leu	Leu	Arg	Gln	Ser	Pro	Thr	Pro
785					790					795					800
His	Gly	Leu	Ser	Leu	Ser	Asp	Leu	Gln	Glu	Ala	Lys	Tyr	Glu	Thr	Phe
				805					810					815	
Ser	Asp	Asp	Pro	Ser	Pro	Gly	Ala	Ile	Asp	Ser	Asn	Asn	Ser	Leu	Ser
			820					825					830		
Glu	Met	Thr	His	Phe	Arg	Pro	Gln	Leu	His	His	Ser	Gly	Asp	Met	Val
		835					840					845			
Phe	Thr	Pro	Glu	Ser	Gly	Leu	Gln	Leu	Arg	Leu	Asn	Glu	Lys	Leu	Gly
	850					855					860				
Thr	Thr	Ala	Ala	Thr	Glu	Leu	Lys	Lys	Leu	Asp	Phe	Lys	Val	Ser	Ser
865					870					875					880
Thr	Ser	Asn	Asn	Leu	Ile	Ser	Thr	Ile	Pro	Ser	Asp	Asn	Leu	Ala	Ala
				885					890					895	

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Gly Thr Asp Asn Thr Ser Ser Leu Gly Pro Pro Ser Met Pro Val His  
 900 905 910

Tyr Asp Ser Gln Leu Asp Thr Thr Leu Phe Gly Lys Lys Ser Ser Pro  
 915 920 925

Leu Thr Glu Ser Gly Gly Pro Leu Ser Leu Ser Glu Glu Asn Asn Asp  
 930 935 940

Ser Lys Leu Leu Glu Ser Gly Leu Met Asn Ser Gln Glu Ser Ser Trp  
 945 950 955 960

Gly Lys Asn Val Ser Ser Thr Glu Ser Gly Arg Leu Phe Lys Gly Lys  
 965 970 975

Arg Ala His Gly Pro Ala Leu Leu Thr Lys Asp Asn Ala Leu Phe Lys  
 980 985 990

Val Ser Ile Ser Leu Leu Lys Thr Asn Lys Thr Ser Asn Asn Ser Ala  
 995 1000 1005

Thr Asn Arg Lys Thr His Ile Asp Gly Pro Ser Leu Leu Ile Glu  
 1010 1015 1020

Asn Ser Pro Ser Val Trp Gln Asn Ile Leu Glu Ser Asp Thr Glu  
 1025 1030 1035

Phe Lys Lys Val Thr Pro Leu Ile His Asp Arg Met Leu Met Asp  
 1040 1045 1050

Lys Asn Ala Thr Ala Leu Arg Leu Asn His Met Ser Asn Lys Thr  
 1055 1060 1065

Thr Ser Ser Lys Asn Met Glu Met Val Gln Gln Lys Lys Glu Gly  
 1070 1075 1080

Pro Ile Pro Pro Asp Ala Gln Asn Pro Asp Met Ser Phe Phe Lys  
 1085 1090 1095

Met Leu Phe Leu Pro Glu Ser Ala Arg Trp Ile Gln Arg Thr His  
 1100 1105 1110

Gly Lys Asn Ser Leu Asn Ser Gly Gln Gly Pro Ser Pro Lys Gln  
 1115 1120 1125

Leu Val Ser Leu Gly Pro Glu Lys Ser Val Glu Gly Gln Asn Phe  
 1130 1135 1140

Leu Ser Glu Lys Asn Lys Val Val Val Gly Lys Gly Glu Phe Thr  
 1145 1150 1155

Lys Asp Val Gly Leu Lys Glu Met Val Phe Pro Ser Ser Arg Asn  
 1160 1165 1170

Leu Phe Leu Thr Asn Leu Asp Asn Leu His Glu Asn Asn Thr His  
 1175 1180 1185

Asn Gln Glu Lys Lys Ile Gln Glu Glu Ile Glu Lys Lys Glu Thr  
 1190 1195 1200

Leu Ile Gln Glu Asn Val Val Leu Pro Gln Ile His Thr Val Thr  
 1205 1210 1215

Gly Thr Lys Asn Phe Met Lys Asn Leu Phe Leu Leu Ser Thr Arg  
 1220 1225 1230

Gln Asn Val Glu Gly Ser Tyr Asp Gly Ala Tyr Ala Pro Val Leu  
 1235 1240 1245

Gln Asp Phe Arg Ser Leu Asn Asp Ser Thr Asn Arg Thr Lys Lys  
 1250 1255 1260

His Thr Ala His Phe Ser Lys Lys Gly Glu Glu Glu Asn Leu Glu  
 1265 1270 1275

Gly Leu Gly Asn Gln Thr Lys Gln Ile Val Glu Lys Tyr Ala Cys  
 1280 1285 1290

Thr Thr Arg Ile Ser Pro Asn Thr Ser Gln Gln Asn Phe Val Thr

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1295	1300	1305
Gln Arg Ser Lys Arg Ala Leu Lys Gln Phe Arg Leu Pro Leu Glu 1310 1315 1320		
Glu Thr Glu Leu Glu Lys Arg Ile Ile Val Asp Asp Thr Ser Thr 1325 1330 1335		
Gln Trp Ser Lys Asn Met Lys His Leu Thr Pro Ser Thr Leu Thr 1340 1345 1350		
Gln Ile Asp Tyr Asn Glu Lys Glu Lys Gly Ala Ile Thr Gln Ser 1355 1360 1365		
Pro Leu Ser Asp Cys Leu Thr Arg Ser His Ser Ile Pro Gln Ala 1370 1375 1380		
Asn Arg Ser Pro Leu Pro Ile Ala Lys Val Ser Ser Phe Pro Ser 1385 1390 1395		
Ile Arg Pro Ile Tyr Leu Thr Arg Val Leu Phe Gln Asp Asn Ser 1400 1405 1410		
Ser His Leu Pro Ala Ala Ser Tyr Arg Lys Lys Asp Ser Gly Val 1415 1420 1425		
Gln Glu Ser Ser His Phe Leu Gln Gly Ala Lys Lys Asn Asn Leu 1430 1435 1440		
Ser Leu Ala Ile Leu Thr Leu Glu Met Thr Gly Asp Gln Arg Glu 1445 1450 1455		
Val Gly Ser Leu Gly Thr Ser Ala Thr Asn Ser Val Thr Tyr Lys 1460 1465 1470		
Lys Val Glu Asn Thr Val Leu Pro Lys Pro Asp Leu Pro Lys Thr 1475 1480 1485		
Ser Gly Lys Val Glu Leu Leu Pro Lys Val His Ile Tyr Gln Lys 1490 1495 1500		
Asp Leu Phe Pro Thr Glu Thr Ser Asn Gly Ser Pro Gly His Leu 1505 1510 1515		
Asp Leu Val Glu Gly Ser Leu Leu Gln Gly Thr Glu Gly Ala Ile 1520 1525 1530		
Lys Trp Asn Glu Ala Asn Arg Pro Gly Lys Val Pro Phe Leu Arg 1535 1540 1545		
Val Ala Thr Glu Ser Ser Ala Lys Thr Pro Ser Lys Leu Leu Asp 1550 1555 1560		
Pro Leu Ala Trp Asp Asn His Tyr Gly Thr Gln Ile Pro Lys Glu 1565 1570 1575		
Glu Trp Lys Ser Gln Glu Lys Ser Pro Glu Lys Thr Ala Phe Lys 1580 1585 1590		
Lys Lys Asp Thr Ile Leu Ser Leu Asn Ala Cys Glu Ser Asn His 1595 1600 1605		
Ala Ile Ala Ala Ile Asn Glu Gly Gln Asn Lys Pro Glu Ile Glu 1610 1615 1620		
Val Thr Trp Ala Lys Gln Gly Arg Thr Glu Arg Leu Cys Ser Gln 1625 1630 1635		
Asn Pro Pro Val Leu Lys Arg His Gln Arg Glu Ile Thr Arg Thr 1640 1645 1650		
Thr Leu Gln Ser Asp Gln Glu Glu Ile Asp Tyr Asp Asp Thr Ile 1655 1660 1665		
Ser Val Glu Met Lys Lys Glu Asp Phe Asp Ile Tyr Asp Glu Asp 1670 1675 1680		
Glu Asn Gln Ser Pro Arg Ser Phe Gln Lys Lys Thr Arg His Tyr 1685 1690 1695		

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Phe	Ile	Ala	Ala	Val	Glu	Arg	Leu	Trp	Asp	Tyr	Gly	Met	Ser	Ser
1700						1705					1710			
Ser	Pro	His	Val	Leu	Arg	Asn	Arg	Ala	Gln	Ser	Gly	Ser	Val	Pro
1715						1720					1725			
Gln	Phe	Lys	Lys	Val	Val	Phe	Gln	Glu	Phe	Thr	Asp	Gly	Ser	Phe
1730						1735					1740			
Thr	Gln	Pro	Leu	Tyr	Arg	Gly	Glu	Leu	Asn	Glu	His	Leu	Gly	Leu
1745						1750					1755			
Leu	Gly	Pro	Tyr	Ile	Arg	Ala	Glu	Val	Glu	Asp	Asn	Ile	Met	Val
1760						1765					1770			
Thr	Phe	Arg	Asn	Gln	Ala	Ser	Arg	Pro	Tyr	Ser	Phe	Tyr	Ser	Ser
1775						1780					1785			
Leu	Ile	Ser	Tyr	Glu	Glu	Asp	Gln	Arg	Gln	Gly	Ala	Glu	Pro	Arg
1790						1795					1800			
Lys	Asn	Phe	Val	Lys	Pro	Asn	Glu	Thr	Lys	Thr	Tyr	Phe	Trp	Lys
1805						1810					1815			
Val	Gln	His	His	Met	Ala	Pro	Thr	Lys	Asp	Glu	Phe	Asp	Cys	Lys
1820						1825					1830			
Ala	Trp	Ala	Tyr	Phe	Ser	Asp	Val	Asp	Leu	Glu	Lys	Asp	Val	His
1835						1840					1845			
Ser	Gly	Leu	Ile	Gly	Pro	Leu	Leu	Val	Cys	His	Thr	Asn	Thr	Leu
1850						1855					1860			
Asn	Pro	Ala	His	Gly	Arg	Gln	Val	Thr	Val	Gln	Glu	Phe	Ala	Leu
1865						1870					1875			
Phe	Phe	Thr	Ile	Phe	Asp	Glu	Thr	Lys	Ser	Trp	Tyr	Phe	Thr	Glu
1880						1885					1890			
Asn	Met	Glu	Arg	Asn	Cys	Arg	Ala	Pro	Cys	Asn	Ile	Gln	Met	Glu
1895						1900					1905			
Asp	Pro	Thr	Phe	Lys	Glu	Asn	Tyr	Arg	Phe	His	Ala	Ile	Asn	Gly
1910						1915					1920			
Tyr	Ile	Met	Asp	Thr	Leu	Pro	Gly	Leu	Val	Met	Ala	Gln	Asp	Gln
1925						1930					1935			
Arg	Ile	Arg	Trp	Tyr	Leu	Leu	Ser	Met	Gly	Ser	Asn	Glu	Asn	Ile
1940						1945					1950			
His	Ser	Ile	His	Phe	Ser	Gly	His	Val	Phe	Thr	Val	Arg	Lys	Lys
1955						1960					1965			
Glu	Glu	Tyr	Lys	Met	Ala	Leu	Tyr	Asn	Leu	Tyr	Pro	Gly	Val	Phe
1970						1975					1980			
Glu	Thr	Val	Glu	Met	Leu	Pro	Ser	Lys	Ala	Gly	Ile	Trp	Arg	Val
1985						1990					1995			
Glu	Cys	Leu	Ile	Gly	Glu	His	Leu	His	Ala	Gly	Met	Ser	Thr	Leu
2000						2005					2010			
Phe	Leu	Val	Tyr	Ser	Asn	Lys	Cys	Gln	Thr	Pro	Leu	Gly	Met	Ala
2015						2020					2025			
Ser	Gly	His	Ile	Arg	Asp	Phe	Gln	Ile	Thr	Ala	Ser	Gly	Gln	Tyr
2030						2035					2040			
Gly	Gln	Trp	Ala	Pro	Lys	Leu	Ala	Arg	Leu	His	Tyr	Ser	Gly	Ser
2045						2050					2055			
Ile	Asn	Ala	Trp	Ser	Thr	Lys	Glu	Pro	Phe	Ser	Trp	Ile	Lys	Val
2060						2065					2070			
Asp	Leu	Leu	Ala	Pro	Met	Ile	Ile	His	Gly	Ile	Lys	Thr	Gln	Gly
2075						2080					2085			
Ala	Arg	Gln	Lys	Phe	Ser	Ser	Leu	Tyr	Ile	Ser	Gln	Phe	Ile	Ile
2090						2095					2100			

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Met	Tyr	Ser	Leu	Asp	Gly	Lys	Lys	Trp	Gln	Thr	Tyr	Arg	Gly	Asn
	2105					2110					2115			
Ser	Thr	Gly	Thr	Leu	Met	Val	Phe	Phe	Gly	Asn	Val	Asp	Ser	Ser
	2120					2125					2130			
Gly	Ile	Lys	His	Asn	Ile	Phe	Asn	Pro	Pro	Ile	Ile	Ala	Arg	Tyr
	2135					2140					2145			
Ile	Arg	Leu	His	Pro	Thr	His	Tyr	Ser	Ile	Arg	Ser	Thr	Leu	Arg
	2150					2155					2160			
Met	Glu	Leu	Met	Gly	Cys	Asp	Leu	Asn	Ser	Cys	Ser	Met	Pro	Leu
	2165					2170					2175			
Gly	Met	Glu	Ser	Lys	Ala	Ile	Ser	Asp	Ala	Gln	Ile	Thr	Ala	Ser
	2180					2185					2190			
Ser	Tyr	Phe	Thr	Asn	Met	Phe	Ala	Thr	Trp	Ser	Pro	Ser	Lys	Ala
	2195					2200					2205			
Arg	Leu	His	Leu	Gln	Gly	Arg	Ser	Asn	Ala	Trp	Arg	Pro	Gln	Val
	2210					2215					2220			
Asn	Asn	Pro	Lys	Glu	Trp	Leu	Gln	Val	Asp	Phe	Gln	Lys	Thr	Met
	2225					2230					2235			
Lys	Val	Thr	Gly	Val	Thr	Thr	Gln	Gly	Val	Lys	Ser	Leu	Leu	Thr
	2240					2245					2250			
Ser	Met	Tyr	Val	Lys	Glu	Phe	Leu	Ile	Ser	Ser	Ser	Gln	Asp	Gly
	2255					2260					2265			
His	Gln	Trp	Thr	Leu	Phe	Phe	Gln	Asn	Gly	Lys	Val	Lys	Val	Phe
	2270					2275					2280			
Gln	Gly	Asn	Gln	Asp	Ser	Phe	Thr	Pro	Val	Val	Asn	Ser	Leu	Asp
	2285					2290					2295			
Pro	Pro	Leu	Leu	Thr	Arg	Tyr	Leu	Arg	Ile	His	Pro	Gln	Ser	Trp
	2300					2305					2310			
Val	His	Gln	Ile	Ala	Leu	Arg	Met	Glu	Val	Leu	Gly	Cys	Glu	Ala
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Gln	Asp	Leu	Tyr											
	2330													

<210> SEQ ID NO 4  
 <211> LENGTH: 5012  
 <212> TYPE: DNA  
 <213> ORGANISM: artificial sequence  
 <220> FEATURE:  
 <223> OTHER INFORMATION: FVIII without domain B

<400> SEQUENCE: 4

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gtgcagtgga actgtcatgg gactatatgc aaagtgatct cggtgagctg cctgtggacg	180
caaggtttgt ttatgcatcc ttttttaaaa tacattgagt atgcttgctt tttagatata	240
gaaatatctg atgctgtctt cttcactaaa ttttgattac atgatttgac agcaatattg	300
aagagtctaa cagccagcac gcaggttggt aagtactgtg ggaacatcac agattttggc	360
tccatgccct aaagagaaat tggctttcag attatttggga ttaaaaacaa agactttctt	420
aagagatgta aaattttcat gatgttttct tttttgctaa aactaaagaa ttaacgcgta	480
ttcttttaca tttcagattt cctcctagag tgccaaaatc ttttccattc aacacctcag	540
tcgtgtacaa aaagactctg tttgtagaat tcacggatca ccttttcaac atcgctaagc	600
caaggccacc ctggatgggt ctgctaggtc ctaccatcca ggctgaggtt tatgatacag	660

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cctactggaa	agcttctgag	ggagctgaat	atgatgatca	gaccagtcaa	agggagaaag	780
aagatgataa	agtcttccct	ggggaagcc	atacatatgt	ctggcaggtc	ctgaaagaga	840
atggccaat	ggcctctgac	ccactgtgcc	ttacctactc	atatctttct	catgtggacc	900
tggtaaaaga	cttgaattca	ggcctcattg	gagccctact	agtatgtaga	gaagggagtc	960
tgccaagga	aaagacacag	accttgcaca	aatttatact	actttttgct	gtatttgatg	1020
aagggaaaag	ttggcactca	gaaacaaaga	actccttgat	gcaggatagg	gatgctgcat	1080
ctgctcgggc	ctggcctaaa	atgcacacag	tcaatggta	tgtaaacagg	tctctgccag	1140
gtctgattgg	atgccacagg	aatcagctct	attggcatgt	gattggaatg	ggcaccactc	1200
ctgaagtga	ctcaatattc	ctcgaaggtc	acacatttct	tgtgaggaac	catcgccagg	1260
cgctcttggg	aatctcgcca	ataactttcc	ttactgctca	aacactcttg	atggaccttg	1320
gacagtttct	actgttttgt	catatctctt	cccaccaaca	tgatggcatg	gaagcttatg	1380
tcaaagtaga	cagctgtcca	gaggaacccc	aactacgaat	gaaaaataat	gaagaagcgg	1440
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<213> ORGANISM: artificial sequence
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<400> SEQUENCE: 5

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		35					40					45			
Arg	Pro	Pro	Trp	Met	Gly	Leu	Leu	Gly	Pro	Thr	Ile	Gln	Ala	Glu	Val
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Tyr	Asp	Thr	Val	Val	Ile	Thr	Leu	Lys	Asn	Met	Ala	Ser	His	Pro	Val
65					70					75					80
Ser	Leu	His	Ala	Val	Gly	Val	Ser	Tyr	Trp	Lys	Ala	Ser	Glu	Gly	Ala
				85					90					95	
Glu	Tyr	Asp	Asp	Gln	Thr	Ser	Gln	Arg	Glu	Lys	Glu	Asp	Asp	Lys	Val
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		115					120					125			
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		130				135					140				
His	Val	Asp	Leu	Val	Lys	Asp	Leu	Asn	Ser	Gly	Leu	Ile	Gly	Ala	Leu
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Leu	Val	Cys	Arg	Glu	Gly	Ser	Leu	Ala	Lys	Glu	Lys	Thr	Gln	Thr	Leu
				165					170					175	
His	Lys	Phe	Ile	Leu	Leu	Phe	Ala	Val	Phe	Asp	Glu	Gly	Lys	Ser	Trp
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His	Ser	Glu	Thr	Lys	Asn	Ser	Leu	Met	Gln	Asp	Arg	Asp	Ala	Ala	Ser
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Val	Ile	Gly	Met	Gly	Thr	Thr	Pro	Glu	Val	His	Ser	Ile	Phe	Leu	Glu
				245					250					255	
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Ile	Gln	Ile	Arg	Ser	Val	Ala	Lys	Lys	His	Pro	Lys	Thr	Trp	Val	His
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Tyr	Ile	Ala	Ala	Glu	Glu	Glu	Asp	Trp	Asp	Tyr	Ala	Pro	Leu	Val	Leu
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Ala	Pro	Asp	Asp	Arg	Ser	Tyr	Lys	Ser	Gln	Tyr	Leu	Asn	Asn	Gly	Pro
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Gln	Arg	Ile	Gly	Arg	Lys	Tyr	Lys	Lys	Val	Arg	Phe	Met	Ala	Tyr	Thr
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Ser Gly Leu Ile Gly Pro Leu Leu Ile Cys Tyr Lys Glu Ser Val Asp  
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Ser Ile Gly Ala Gln Thr Asp Phe Leu Ser Val Phe Phe Ser Gly Tyr  
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Phe Ser Gly Glu Thr Val Phe Met Ser Met Glu Asn Pro Gly Leu Trp  
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 675 680 685

Leu Leu Lys Val Ser Ser Cys Asp Lys Asn Thr Gly Asp Tyr Tyr Glu  
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Asp Ser Tyr Glu Asp Ile Ser Ala Tyr Leu Leu Ser Lys Asn Asn Ala  
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Lys Lys Glu Asp Phe Asp Ile Tyr Asp Glu Asp Glu Asn Gln Ser Pro  
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Arg Ser Phe Gln Lys Lys Thr Arg His Tyr Phe Ile Ala Ala Val Glu  
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Arg Leu Trp Asp Tyr Gly Met Ser Ser Ser Pro His Val Leu Arg Asn  
 785 790 795 800

Arg Ala Gln Ser Gly Ser Val Pro Gln Phe Lys Lys Val Val Phe Gln  
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Glu Phe Thr Asp Gly Ser Phe Thr Gln Pro Leu Tyr Arg Gly Glu Leu  
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Asn Glu His Leu Gly Leu Leu Gly Pro Tyr Ile Arg Ala Glu Val Glu  
 835 840 845

Asp Asn Ile Met Val Thr Phe Arg Asn Gln Ala Ser Arg Pro Tyr Ser  
 850 855 860

Phe Tyr Ser Ser Leu Ile Ser Tyr Glu Glu Asp Gln Arg Gln Gly Ala

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Lys Ala Trp Ala Tyr Phe Ser Asp Val Asp Leu Glu Lys Asp Val His	915	920	925
Ser Gly Leu Ile Gly Pro Leu Leu Val Cys His Thr Asn Thr Leu Asn	930	935	940
Pro Ala His Gly Arg Gln Val Thr Val Gln Glu Phe Ala Leu Phe Phe	945	950	955
Thr Ile Phe Asp Glu Thr Lys Ser Trp Tyr Phe Thr Glu Asn Met Glu	965	970	975
Arg Asn Cys Arg Ala Pro Cys Asn Ile Gln Met Glu Asp Pro Thr Phe	980	985	990
Lys Glu Asn Tyr Arg Phe His Ala Ile Asn Gly Tyr Ile Met Asp Thr	995	1000	1005
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Ser Gly His Val Phe Thr Val Arg Lys Lys Glu Glu Tyr Lys Met	1040	1045	1050
Ala Leu Tyr Asn Leu Tyr Pro Gly Val Phe Glu Thr Val Glu Met	1055	1060	1065
Leu Pro Ser Lys Ala Gly Ile Trp Arg Val Glu Cys Leu Ile Gly	1070	1075	1080
Glu His Leu His Ala Gly Met Ser Thr Leu Phe Leu Val Tyr Ser	1085	1090	1095
Asn Lys Cys Gln Thr Pro Leu Gly Met Ala Ser Gly His Ile Arg	1100	1105	1110
Asp Phe Gln Ile Thr Ala Ser Gly Gln Tyr Gly Gln Trp Ala Pro	1115	1120	1125
Lys Leu Ala Arg Leu His Tyr Ser Gly Ser Ile Asn Ala Trp Ser	1130	1135	1140
Thr Lys Glu Pro Phe Ser Trp Ile Lys Val Asp Leu Leu Ala Pro	1145	1150	1155
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Met Val Phe Phe Gly Asn Val Asp Ser Ser Gly Ile Lys His Asn	1205	1210	1215
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Cys Asp Leu Asn Ser Cys Ser Met Pro Leu Gly Met Glu Ser Lys	1250	1255	1260
Ala Ile Ser Asp Ala Gln Ile Thr Ala Ser Ser Tyr Phe Thr Asn	1265	1270	1275

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Glu	Phe	Leu	Ile	Ser	Ser	Ser	Gln	Asp	Gly	His	Gln	Trp	Thr	Leu
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Phe	Phe	Gln	Asn	Gly	Lys	Val	Lys	Val	Phe	Gln	Gly	Asn	Gln	Asp
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Arg	Tyr	Leu	Arg	Ile	His	Pro	Gln	Ser	Trp	Val	His	Gln	Ile	Ala
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Leu	Arg	Met	Glu	Val	Leu	Gly	Cys	Glu	Ala	Gln	Asp	Leu	Tyr	
	1400					1405					1410			

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The invention claimed is:

**1.** An isolated human coagulation factor VIII (FVIII) variant comprising a substitution of the amino acid at position 462 of SEQ ID NO: 3, wherein said variant has decreased antigenicity towards inhibitory antibodies as compared to natural human FVIII, retains procoagulant activity and, optionally, totally or partially lacks the domain B, and wherein the polypeptide sequence of the variant differs from SEQ ID NO: 3 by 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 or 15 substitutions, without including the optional total or partial deletion of the domain B.

**2.** The isolated human coagulation FVIII variant according to claim 1, wherein said variant comprises a single amino acid substitution.

**3.** The isolated human coagulation FVIII variant according to claim 1, wherein said variant further comprises a substitution of at least one amino acid selected from the group consisting of the amino acids at position 2202 and 437 of SEQ ID NO: 3.

**4.** The isolated human coagulation FVIII variant according to claim 1, wherein said variant contains a combination of two substitutions selected from the group consisting of the amino acids at positions 409+462, 462+507 and, 462+629 of SEQ ID NO: 3.

**5.** The isolated human coagulation FVIII variant according to claim 1, wherein said variant contains a combination of three substitutions selected from the group consisting of the amino acids at positions 409+462+507, 462+507+629, and 409+462+629 of SEQ ID NO: 3.

**6.** The isolated human coagulation FVIII variant according to claim 1, wherein said variant contains a combination of four substitutions of the amino acids at positions 409, 462, 507 and 629 of SEQ ID NO: 3.

**7.** The isolated human coagulation FVIII variant according to claim 1, wherein said variant further comprises a substitution of at least one amino acid selected from the group consisting of the amino acids at positions 2177, 2183, 2186, 2191, 2196, 2204, 2205, 2213, 2217, 2235, 2258, 2264, 2268 and 2269 of SEQ ID NO: 3.

**8.** The isolated human coagulation FVIII variant according to claim 1, wherein said variant further comprises a substitu-

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tion of at least one amino acid selected from the group consisting of the amino acids at positions 2175, 2199, 2200, 2215, 2251, 2252 and 2278 of SEQ ID NO: 3.

**9.** The isolated human coagulation FVIII variant according to claim 1, wherein the amino acid is substituted by an amino acid selected from an Alanine, a Methionine, a Serine, or a Glycine.

**10.** The isolated human coagulation FVIII variant according to claim 9, wherein the substituted amino acid is an Alanine.

**11.** The isolated human coagulation FVIII variant according to claim 1, said human coagulation FVIII variant comprising a substitution of the amino acid at position 462 of SEQ ID NO: 3 and said substitution of 1 to 15 amino acids at a position in SEQ ID NO: 3 is selected from the group consisting of 400, 403, 409, 414, 421, 437, 486, 493, 494, 496, 507, 518, 562, 629, 2175, 2177, 2183, 2186, 2191, 2196, 2199, 2200, 2202, 2204, 2205, 2206, 2212, 2213, 2215, 2217, 2226, 2235, 2244, 2251, 2252, 2258, 2261, 2264, 2268, 2269, 2275, 2278, 2280, 2281, 2282, 2289, 2294, 2311, 2312 and 2316, wherein said variant has decreased antigenicity towards inhibitory antibodies as compared to natural human FVIII, retains procoagulant activity and, optionally, totally or partially lacks the domain B.

**12.** A pharmaceutical composition comprising the isolated human coagulation FVIII variant according to claim 1 and a pharmaceutically acceptable carrier or excipient.

**13.** A method for treating hemophilia A in a patient, comprising administering the isolated human coagulation FVIII variant according to claim 1 to said patient.

**14.** The method according to claim 13, wherein the patient to be treated is a hemophiliac patient with inhibitors.

**15.** The method according to claim 13, wherein the patient to be treated is a hemophiliac patient before the development of inhibitors.

**16.** A method for determining an inhibitor type in a patient with hemophilia A comprising performing a recognition test of inhibitory antibodies contained in a serum sample from the patient on one or more isolated human coagulation FVIII variants according to claim 1.

\* \* \* \* \*

UNITED STATES PATENT AND TRADEMARK OFFICE  
**CERTIFICATE OF CORRECTION**

PATENT NO. : 8,623,824 B2  
APPLICATION NO. : 12/528379  
DATED : January 7, 2014  
INVENTOR(S) : Didier Saboulard et al.

Page 1 of 6

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

In the Specification

Column 3,

Line 2, "FVIII variants" should read --FVIII variants--.

Line 8, "US20021165177;" should read --US2002/165177;--.

Column 4,

Line 60, "FVIII activity" should read --FVIII activity--.

Column 5,

Line 10, "antibodies not" should read --antibodies do not--.

Column 6,

Line 61, "group speculate" should read --group speculates--.

Column 7,

Line 49, "five six," should read --five, six,--.

Column 10,

Line 5, "(TD, GC, PR, SL and FS)" should read --(TD (Figure 6C), GC (Figure 6B), PR (Figure 6D), SL (Figure 6E) and FS (Figure 6A))--.

Lines 11-12, "(GMA012) and a rabbit polyclonal antibody." should read --(GMA012, Figure 7A) and a rabbit polyclonal antibody (Figure 7B).--.

Lines 15-16, "(ESH4) and anti-A2 domain antibody (GMA012)." should read --(ESH4, Figure 8B) and anti-A2 domain antibody (GMA012, Figure 8A).--.

Lines 24-25, "(TD, GC, SL and FS measured by Bethesda assay.

FIGS. 12-14: Primary screen results; list of 158 Alanine"

should read

Signed and Sealed this  
Fifteenth Day of September, 2015



Michelle K. Lee  
Director of the United States Patent and Trademark Office

--(TD (Figure 11A), GC (Figure 11B), SL (Figure 11D) and FS (Figure 11C)) measured by Bethesda assay.

Residual activity, determined after incubation with inhibitory antibodies, is divided by remained activity after incubation with a non-immune antibody to give the residual activity percentage.

FIGS. 12-14: Primary screen results; list of 158 Alanine--.

Column 10, Line 42 through Column 14, Line 47,

“**FIG. 21:** Chromogenic specific activities and abolition to inhibition percentages towards inhibitory antibodies of six double A2 mutants from sera of four hemophiliac patients TD, GC, SL and PR.

#### Description of the invention

The present invention provides a solution to resolve a serious complication that occurs in 30% of hemophilia A patients treated with recombinant FVIII: the development of an immune response induced by the treatment and directed against the exogenous recombinant FVIII. The solution provided consists in generating recombinant human FVIII molecules having decreased antigenicity of the epitopes usually recognized by inhibitory antibodies. The FVIII variants of the invention have lost one or more epitopes usually recognized by said antibodies.

The present invention provides other solutions consisting in generating human FVIII variants having an improved specific activity as compared to natural FVIII.

Lastly, the present invention provides with FVIII variants having a greater capacity to be secreted, which is interesting for the production of recombinant FVIII and in a potential gene therapy.

The different properties conferred by the mutations in these variants may be of major interest in combination. In a non-limiting example, mutations which confer a specific activity improvement of a variant could compensate an optional relative loss of activity in variants whose mutations confer a abolition to inhibition by inhibitory antibodies and being therefore less antigenic. In another non-limiting example, mutations which confer a higher capacity to be secreted may interesting in combination with mutations conferring an abolition to inhibition by inhibitory antibodies, by allowing, for example, to compensate a optional relative loss of secretion of said less antigenic mutants.

In the present document, the following terminology is used to designate a substitution: **5409A** indicates the substitution of the serine residue at position **409** of SEQ ID No. 3 by an alanine. Substitution refers to the replacement of an amino acid residue by another one selected from the other 19 amino acids or by a non-naturally occurring amino acid. The terms “substitution” and “mutation” are interchangeable. The sign “+” indicates a combination of substitutions.

“Comprise” means that the variant or the fragment thereof has one or more substitutions such as indicated with reference to SEQ ID No. 3, but that the variant or the fragment thereof may have other modifications, particularly substitutions, deletions or insertions.

the chromogenic assay mentioned above. This assay was also performed on the robotic platform of the National Hemophilia Treatment Center (Hospices Civils de Lyon). The chromogenic activity of the **158** selected Alanine mutants was carried out with the Coamatic Factor VIII kit (Chromogenix, Instrumentation Laboratory, Milan, Italy) according to the supplier’s instructions. Briefly, culture supernatants (50 µl) were diluted in the dilution buffer provided and preincubated at 37° C. for 4 min. The reaction medium (50 µl), preheated at 37° C., was then added for 4 min, after which 50 µl of development medium at 37° C. were added. The formation of product over time was  
Column 10, Line 42 through Column 14, Line 47 cont’d.

measured immediately on a spectrophotometer at 405 nm after shaking the microtiter plate. Product formation is expressed as mUOD/min. When values were greater than 200 mUOD/min, the assay was repeated using a higher dilution.

FIGS. 12-14 show the activities of the 158 mutants which retained more than 50% of non-mutated FVIII activity. Said 158 mutants were selected for the secondary screening.

Example 4: Secondary screen: Evaluation of loss of antigenicity towards human FVIII inhibitory antibodies

The secondary screen correlates to an assay similar to the Bethesda assay, carried out as described below on the 158 mutants selected following the primary screening; said assay comprises a step of contacting a inhibitory serum (or antibody) with a FVIII molecule to be tested or a reference standard and a step of measuring FVIII coagulant activity by chromometric assay.

Culture supernatants obtained after 48 h of contact with COS cells transfected by different FVIII constructs were used. Said supernatants were produced in complete medium [(IMDM, Invitrogen), 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin]. Supernatants were diluted in fresh complete medium to obtain a final chromometric activity comprised in the range of about 10-20% (1 FVIII unit = 100% activity = 200 ng/ml). The culture supernatant diluted or not (140  $\mu$ l) was added to 150  $\mu$ l of FVIII-depleted human plasma (Stago, Asnieres, France). An antibody dilution (10  $\mu$ l) was then added to the mix. These antibodies are IgG fractions purified on protein A- from hemophiliac patients with inhibitors. An IgG fraction from a non-hemophiliac control was similarly obtained.

Bethesda inhibitor titers were identical to the inhibitory activity from the plasma. The purification protocol therefore did not affect the inhibitory activity of the antibodies. The antibodies were first diluted in fresh complete medium, the measurement being carried out either with a fixed antibody dilution or with serial dilutions. The fixed antibody concentration which was used was that which produced 50% inhibition of a recombinant FVIII standard solution with 12.5% activity. Samples were incubated in a 37° C. water-bath for 1h30. Coagulant activity was then determined on a MDA-II apparatus (BioMérieux, Marcy-l'Etoile) and compared to that of a standard curve established from an identical FVIII stably produced in the CHO cell line. Results are expressed as a percentage which represents the abolition to inhibition of coagulant activity of a given mutant by inhibitory antibodies from a patient's serum. Said percentage was calculated as shown in Figure 5 for the FVIII mutant E518A. Abolition to inhibition expressed is a percentage =  $-(b-a)/a \times 100$ ; where "a" is the percentage residual activity of the WT (serum + IgG / serum - IgG) and "b" is the percentage residual activity of the mutant (serum + IgG / serum - IgG).

FIGS. 15-18 show for 30 single mutants the percentages of abolition to inhibition for sera from five hemophiliac patients. Said mutants were selected in the secondary screen of the 158 mutants selected in the primary screen. Several mutants show a high percentage of abolition to inhibition with certain sera, such as mutant 2316 for sera TD and SL, mutant 2294 for serum GC, mutant 403 for serum FS and mutant 2275 for serum PR.

Patients' sera were selected for their high Bethesda titers (greater than 10 BU) and their different inhibitor profiles. These patients can no longer be treated with FVIII injections and need bypassing agents. Thus, obtaining FVIII Alanine mutants which abolish, even partially, the inhibition of FVIII activity by the inhibitory antibodies of one of these patients, is a major step forward to the future approaches of treating hemophiliac patients with inhibitors. The different data obtained on a large number of mutants as well as the different sera tested will make it possible to create  
Column 10, Line 42 through Column 14, Line 47 cont'd.

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combinations of mutations leading to an improved FVIII which can avoid a majority of inhibitory antibodies while retaining its procoagulant activity.

The reproducibility of FVIII expression level related to transfections was controlled by following the specific activity of wild-type FVIII. Indeed, specific activities calculated from antigen determinations (Stago commercial ELISA kit) were identical for wild-type FVIIs produced in different transfections. Likewise, antigen concentrations were determined for mutants having retained at least **50%** of wild-type FVIII activity and their specific activity was determinate throw. Specific activity corresponds to raw activity measured in the chromogenic assay (mUOD/min) relative to protein concentration (ng/ml) obtained with an ELISA kit (Stago FVIII kit). Figure **19** shows comparative data of raw and specific activities of **30** mutants selected in the secondary screen.

The eight FVIII Alanine mutants **2175, 2199, 2200, 2215, 2251, 2252, 2278** and **2316** displayed a far above average capacity to be secreted in the COS cell production medium used in the scope of the present invention. FIG. 3 depicts the data obtained for these eight mutants. Raw coagulant activity of these mutants was determined by chromogenic assay. Their concentration was approximately two to four times higher than that of wild-type FVIII. This property is interesting for producing recombinant FVIII and might make it possible to lower production costs of a new generation FVIII. Also, it might be advantageous in a gene therapy for hemophiliac patients. Moreover, these mutations which confer a greater capacity to be secreted may be of major interest in combination with mutations conferring abolition to inhibition by inhibitory antibodies, by allowing, for example, to compensate an optional relative loss of secretion of said less antigenic mutants.

The 15 mutants **2177, 2183, 2186, 2191, 2196, 2204, 2205, 2206, 2213, 2217, 2235, 2258, 2264, 2268** and **2269** displayed far higher specific activity than wild-type FVIII, while maintaining a high production level, around to that of wild-type FVIII (concentration greater than 10 ng/ml). The specific activities of these 15mutants are given in FIG. 4. Raw coagulant activity of these mutants was determined by chromogenic assay. This property is interesting because it would allow smaller or less frequent doses of FVIII to be injected in patients. Moreover, these mutations which confer a higher specific activity might be of major interest in combination with mutations conferring abolition to inhibition by inhibitory antibodies, by allowing to compensate an optional relative loss of activity of said less antigenic mutants.

Example 5: Selection and combination of the best single mutants selected in the secondary screen

Among the 30 single mutants selected in the secondary screen, eight were chosen in order to combine their respective mutations, to obtain a cumulative/additive effect of remarkable properties of each. The selection criteria for these mutants were complex and considered the following parameters:

- at least 25% abolition to inhibition for at least one of the test sera from hemophiliac patients with inhibitors;

- raw coagulant activity at least **100%** relative to non-mutated FVIII; and

- reproducibly good level of expression.

The eight selected mutants were mutants **409, 462, 507** and **629** in the A2 domain and mutants **2289, 2294, 2312** and **2316** in the C2 domain. As noted earlier, the selection criterion considered of a high specific activity (coagulant activity relative to expression level), as shown in FIG. **19**. This specific activity level had to be constant in the different experiments.

The 28 double mutants resulting from the combination of the eight single mutations **409, 462, 507, 629, 2289, 2294, 2312** and **2316** (six A2 double mutants +six C2 double mutants +sixteen A2-C2  
Column 10, Line 42 through Column 14, Line 47 cont'd.



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double mutants presented in FIG. 20) were constructed by mutagenesis methods known to one skilled in the art. These mutants were transiently expressed in COS-7 mammalian cells as described in Example 2. Their expression level and their activity level were determined as described in the previous examples, respectively by ELISA and chromogenic assay (mUOD/min). These 28 mutants were then assessed for their abolition to inhibition by antibodies from hemophiliac patients. The A2 double mutants displayed a significant abolition to inhibition for one or all of the antibodies from the patients' sera, whereas the combinations containing C2 domain mutations (six C2 double mutants + sixteen A2-C2 double mutants) displayed an insignificant or null abolition to inhibition.

FIG. 21 shows the specific activities of the six A2 double mutants and their percentage of abolition to inhibition by sera from four hemophiliac patients TD, GC, SL and PR calculated as in Example 4. Especially preferred double mutants significantly abolished antibodies from a minimum of three over the four patients. This illustrates the cumulative effect of the four single mutations in the A2 domain. The choice was therefore based on the combination of the four mutations 409, 507, 462 and 629. Triple mutants and the quadruple mutant comprising these four mutations 409, 507, 462 and 629 were also constructed.

Residual activity, determined after incubation with inhibitory antibodies, is divided by remained activity after incubation with a non-immune antibody to give the residual activity percentage.

Table 1: Primary screen results; list of 158 Alanine mutants selected for secondary screening, having retained at least 50% of raw activity relative to non-mutated FVIII activity.

Table 2: Secondary screening: Bethesda assays on 30 mutants displaying modified antigenicity towards sera from five hemophiliac patients with inhibitors. Results are expressed as the abolition to inhibition percentage for each mutant as exemplified in FIG. 5.

Table 3: Comparison of specific activity and raw activity relative to non-mutated FVIII activity for the 30 mutants displaying modified antigenicity towards sera from five hemophiliac patients with inhibitors.

Table 4: List of all FVIII double mutants produced from the eight single mutants FVIII409A, FVIII462A, FVIII507A, FVIII629A, FVIII2289A, FVIII2294A, FVIII2312A and FVIII2316A.

Table 5: Chromogenic specific activities and abolition to inhibition percentages towards inhibitory antibodies of six double A2 mutants from sera of four hemophiliac patients TD, GC, SL and PR.

## DESCRIPTION OF THE INVENTION"

should read

--FIG. 21: Chromogenic specific activities and abolition to inhibition percentages towards inhibitory antibodies of six double A2 mutants from sera of four hemophiliac patients TD, GC, SL and PR.

## DESCRIPTION OF THE INVENTION--.

Column 27,

Line 44, "responsible of its" should read --responsible for its--.

Column 28,

Line 20, "from a patient" should read --from one patient--.

Column 31,

Line 48, "of a continuously use" should read --of a continuous use--.

Lines 55-56, "might be encompass for a" should read --might encompass a--.

Column 32,

Line 61, "propose to use of the" should read --propose to use the--.

Lines 66-67, "decrease for the control" should read --decrease the control--.

Column 33,

Line 55, "consisted in" should read --consisted of--.

Column 35,

Line 33, "Table 1 shows" should read --Figures 12-14 show--.

Column 36,

Line 19, "Table 2 shows" should read --Figures 15-18 show--.

Line 52, "Table 3 shows" should read --Figure 19 shows--.

Column 37,

Line 41, "shown in Table 3." should read --shown in Figure 19.--.

Line 46, "in Table 4)" should read --in Figure 20)--.

Line 60, "Table 5 shows" should read --Figure 21 shows--.

Column 39,

Line 35, "about 27±1" should read --about 27 ± 11--.

Line 65, "added (Iris" should read --added (Tris--.